THE LOSS OF FLUID AND PROTEIN FROM THE BLOOD DURING A SYSTEMIC RISE OF VENOUS PRESSURE PRODUCED BY REPEATED VALSALVA MANEUVERS IN MAN [1]

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It is well known that protein-poor fluid moves from the vascular to the extravascular space during venous congestion of the forearm or lower extremity (1–5) and during a more generalized increase of venous pressure such as is induced by quiet standing (6, 7) or by pressure breathing (8, 9). No quantitative comparison has been made, however, between local and generalized congestion as to the quantity and duration of capillary filtration per unit increase of venous pressure.

Our interest in the role played by generalized capillary filtration in the pathophysiology of congestive heart failure and in postpartum hemoconcentration (10) stimulated the following experiments in which venous pressure was increased by repeated performance of Valsalva maneuvers for half-hour periods by healthy subjects. Fluid losses were calculated from changes in venous hematocrit. It will be shown *a*) that in the first 10 minutes the unit rate of filtration is about the same for generalized as for local congestion, but *b*) that the rate subsequently falls off much more abruptly when the filtration area and the early fluid loss are larger than when only the forearm is involved.

PROCEDURE, METHODS AND CALCULATIONS

The venous pressure manometer consisted of glass tubing (inside diameter, 3 mm.) attached to a scale with zero 10 cm. above the table top. The rubber tubing between manometer and vein was interrupted by a T-tube leading from a calibrated 120 ml. reservoir. The entire system was filled with heparinized saline. Manipulation of reservoir level and connecting pinchcocks permitted rapid adjustment of the fluid column in the manometer to match the fluctuations of venous pressure and prevented regurgitation of blood or unnecessary infusion of saline during and after Valsalva maneuvers.

The subjects were healthy [2] men and women under the age of 45. They came directly to the laboratory in the morning, usually without breakfast, and reclined for at least 45 minutes to allow stabilization of postural changes in fluid distribution before observations were begun. An indwelling 18 gauge needle was then placed in the antecubital vein of each arm. One was used for measurements of pressure and injection of dye, and the other for sampling. To keep it patent, the latter was connected to a 20 ml. syringe containing heparinized 0.9 per cent saline so that minimal amounts of blood and saline could be exchanged (0.1 to 0.2 ml.). Before collection of any sample, 3 ml. of blood was discarded.

The subjects rested for another 10 to 30 minutes while three or four blood samples were collected and three or four readings of venous pressure were recorded, and in one experiment on each subject plasma volume was determined with T-1824. A series of Valsalva maneuvers was then performed. Readings of venous pressure were called out by an observer, while the subject, lying on his back with legs flexed, attempted to raise the pressure as high as possible by tensing his abdomen and making respiratory efforts against his closed glottis. Efforts lasted 20 out of every 30 seconds. Each fifth minute was devoted to rest and collection of blood samples (see Figure 1). The manometer was read every five seconds. Observations continued during 30 minutes of recovery. The volumes of saline delivered and of blood withdrawn were recorded.

Samples were collected in oiled syringes to minimize hemolysis. Three ml. of each sample was transferred to a tube containing the appropriate amount of Wintrobe's anticoagulant mixture; the remainder was allowed to clot. Wintrobe hematocrit tubes were filled in duplicate immediately after the procedure, covered with a drop of mineral oil, and centrifuged for 30 minutes at 3,000 rpm. The buffy coat was included in the estimated height of the cell column, and no correction was applied for trapped plasma. Serum proteins were estimated by the falling drop method of Kagan (11); falling times were determined in triplicate. Optical densities of

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[2] A. McL. had had amputations of one leg at the ankle and the other at the junction of the middle and lower third of the foreleg.

the serum were determined with an Evelyn microcolorimeter.

The venous pressure readings taken during the 60 minutes of effort and recovery were plotted on coordinate paper, and the average of the resting readings was drawn as the baseline. The mean increase or decrease of pressure from resting level was then determined by planimetry for each five minute period.

The average value for the three resting samples was used as the "initial" value for both hematocrit and serum protein concentration. Initial plasma volume, when measured, was calculated from the average of the optical densities of samples taken 10 and 15 minutes after injecting the dye. Initial whole blood and red cell volumes were calculated from this value and the initial hematocrit. In experiments where plasma volume was not measured, plasma and whole blood volumes were calculated from the initial hematocrit and the red cell volume as determined in the companion experiment on the same subject.

For each successive sampling period, the initial values were corrected for the losses of cells, plasma and total circulating protein caused by sampling, using the hematocrit and protein values of the preceding sample. In this way "predicted" values for hematocrit and protein concentrations were obtained for comparison with the observed values for the same period. A second set of predicted values was estimated on the assumption that the plasma volume had been expanded by the exact amount of the infused saline. The formulas introduced by Landis, Jonas, Angevine and Erb (2) were used to calculate the apparent changes of plasma volume, of total circulating protein and of the protein content of the capillary filtrate. Details of the calculations and an analysis of the reliability of the results appear in the Appendix.

Because the volumes of saline infused during the Valsalva experiments were relatively large in relation to the observed filtration volumes, 10 experiments were performed in which comparable volumes of saline were infused, but Valsalva maneuvers omitted. Each healthy subject rested for at least 45 minutes, after which two or three samples were taken at 5 minute intervals from an 18 gauge needle kept open with a very slow drip of saline. Saline was then infused at an approximately constant rate for 30 minutes to achieve totals of 150 to 210 ml. A 5 ml. sample was taken every five minutes after 3 ml. of blood had been discarded. The hematocrit of each sample was determined in duplicate.

RESULTS

1. Antecubital venous pressure

Figure 1 shows two examples of the curves obtained by plotting manometer readings. The rise of pressure was rapid at first and became slower toward the end of the 20 seconds of effort. With cessation of the effort, pressure fell abruptly to

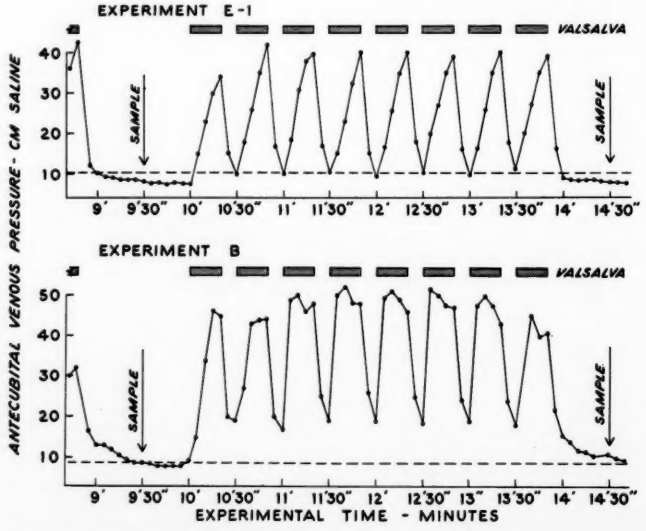

FIG. 1. TWO EXAMPLES OF VENOUS PRESSURE CURVES OBTAINED BY PLOTTING SALINE MANOMETER READINGS DURING VALSALVA MANEUVERS AND DURING REST

Manometer zero 10 cm. above the skin of the back. Dotted lines show average pressures during initial rest period.

TABLE I

Summary of Valsalva experiments

Expt. no.	Date 1947	Subject	Sex	Weight	Plasma volume	Volume of saline added	Volume of blood samples	Maximum venous pressure*
				Kg.	*ml.*	*ml.*	*ml.*	*cm. saline*
A-1	11/29	J. H.	M			210	7	46
A-2	12/9	J. H.	M	80.0	3,360	155	15	41
B	12/16	F. L.	M	72.8	3,460	130	15	42
C	12/2	J. S.	M	76.5	3,197	170	15	45
D-1	11/25	A. McL.	M			142	7	32
D-2	12/11	A. McL.	M	56.6	2,970	120	15	30
E-1	11/28	E. B.	F			160	7	28
E-2	12/18	E. B.	F	60.0	3,100	135	15	28
F-1	12/4	B. F.	F			100	7	27
F-2	12/30	B. F.	F	58.5	2,505	120	15	23

* Average of the maxima reached during Valsalva maneuvers minus the mean resting pressure.

near the original baseline. Table I shows the average peak pressures attained. Some subjects achieved more effective increases of pressure than others, but performance was fairly uniform in each experiment. Average increases of between 10.4 and 20.8 cm. H_2O were sustained during the half-hour periods of Valsalva maneuvers (see Table III and Figure 2). Venous pressure always remained below the initial resting level during the entire 30 minutes of recovery.

TABLE II

Effects of small saline infusions on the plasma volume during rest

Subject	Sex	Weight	Time	Hematocrit		Volume of saline infused	Apparent excess (+) or deficit (−) of plasma
				Predicted	Observed		
		Kg.	*min.*	%	%	*ml.*	(% *of infused volume*)
H. R.	M	70	10	48.1	47.6	60	+ 78
			30	46.7	46.9	180	− 10
R. W.	M	82	10	41.9	42.1	60	− 41
			30	39.9	41.0	180	− 77
S. H.	M	65	10	48.9	48.2	60	+ 97
			30	47.3	47.1	180	+ 10
P. H.	M	81	10	47.2	45.9	60	+210
			30	46.1	46.4	180	− 17
D. Z.	M	73	10	47.8	47.7	70	+ 7
			30	46.5	47.2	210	− 31
J. K.	M	87	10	40.3	40.3	70	0
			30	39.0	40.2	210	− 58
E. B.	F	66	10	42.6	43.3	75	− 87
			30	41.4	42.6	200	− 58
M. D.	F	54	10	38.4	39.1	50	−129
			30	37.2	38.5	150	− 93
M. S.	F	47	10	41.4	41.0	50	+ 39
			30	40.1	40.5	150	− 22
M. J.	F	62	10	40.3	40.9	60	− 71
			30	39.3	40.5	180	− 70
						Average at 10 minutes	+ 10.3 (S.D. = 95)
						Average at 30 minutes	− 44 (S.D. = 33)

2. Experiments in which no Valsalva maneuvers were performed

The amounts of saline infused, 2.2 to 3.2 ml. per Kg. of body weight, were similar in magnitude to those used in the Valsalva experiments. The "predicted" hematocrits shown in Table II represent the average resting values corrected for sampling losses and for the infused saline. Thus, the calculated excesses or deficits represent differences between the apparent changes in plasma volume and the expected changes if all the saline had remained intravascular. The results were extremely variable. At the time of the 10 minute sample more fluid appeared to have been added to the plasma than had been injected in the majority of experiments. At 30 minutes, after infusion of 150 to 210 ml., evidence in all but one experiment indicated that some portion of the fluid had left the blood. The average loss of 44 per cent was derived from values ranging between a loss of 93 per cent of and a gain of 10 per cent over the injected volume.

3. Hemoconcentration during the period of Valsalva efforts as calculated from hematocrit

Because the fate of the saline introduced during the experiments could not be determined exactly (see above), calculations were made in two ways: *a*) without consideration for the saline, and *b*) assuming that plasma volume was expanded progressively by the saline. Values corrected for saline will henceforth be labeled "cs" and uncorrected values "ucs." When correction was made for saline, the predicted hematocrit was always lower and the apparent filtration volume greater than when the saline was left out of consideration. Figure 2 shows the results of two experiments in which the increments of venous pressure were about the same. It shows the similarity between curves obtained by plotting ucs and cs values. In every experiment the fluid loss as calculated from hematocrits was rapid at first, became progressively slower, and ceased after 10 to 20 minutes. In six experiments fluid was regained during the latter part of the period of increased venous pressure (see Experiment A-2, Figure 2).

In general, the largest subjects and those who maintained the highest increments of venous pressure filtered the most fluid. The three heaviest subjects, A, B and C, maintained venous pressures 17 to 22 cm. above resting. During the first nine and a half minutes their fluid losses were 333 to 501 ml. (5.6 to 8.1 per cent of initial blood volume cs). The two females, E and F, maintained venous pressures only 9 to 13 cm. above resting and lost only 3.1 to 3.9 per cent of blood volume (see Table III).

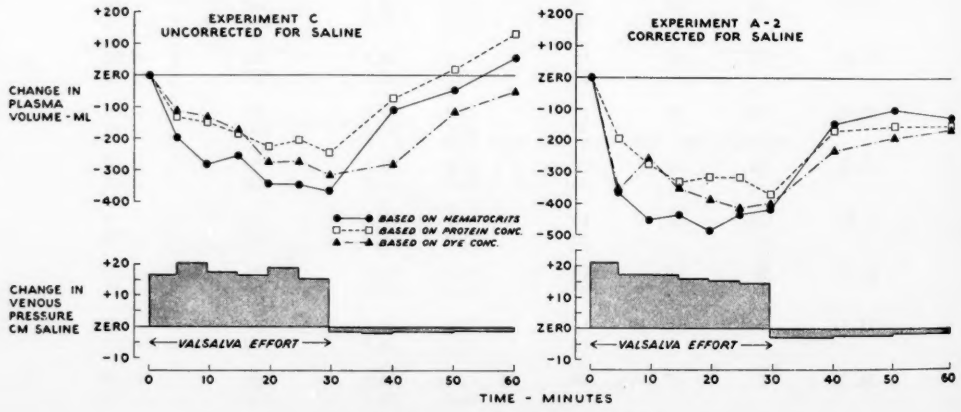

FIG. 2. SUMMARY OF TWO EXPERIMENTS

Below are the average changes of venous pressure during Valsalva efforts and during recovery, as determined by planimetry of pressure curves for each five minutes. Apparent changes in plasma volume, as calculated from hematocrits, serum proteins and optical densities, appear above. Values for optical density have been calculated as though dye were leaving the blood continuously at a rate of 10 per cent per hour.

TABLE III

Results at 9½ and 29½ minutes in Valsalva experiments

Expt. no.	Time	Increase in venous pressure*	Hematocrit			Serum protein conc.			Volume filtered§		Blood volume lost§		Unit rate of filtration§		Change in total circulating protein‖
			Observed	Predicted		Observed	Predicted								
				cs†	ucs‡		cs	ucs	cs	ucs	cs	ucs	cs	ucs	cs
	min.	*cm. saline*	%	%		*Gm. %*	*Gm. %*		*ml.*		%		*ml./min./cm./Kg.*		*Gm.*
A-1	9½	19.1	49.85	47.04	47.61	6.322	5.851	5.989	333	262	5.7	4.5	0.023	0.018	− 6.5
	29½	20.1	49.75	45.95	47.62	6.391	5.603	5.989	460	250	7.8	4.3	0.010	0.005	− 5.8
A-2	9½	19.2	48.50	44.93	45.31	6.305	5.796	5.886	454	402	7.4	6.6	0.033	0.028	−11.4
	29½	16.9	47.35	44.17	45.35	6.343	5.660	5.882	417	262	6.8	4.3	0.010	0.006	− 2.9
B	9½	20.8	47.45	43.60	43.91	6.567	5.938	6.010	501	459	8.1	7.4	0.035	0.032	−11.0
	29½	20.8	48.30	42.96	43.88	6.764	5.785	6.005	687	556	11.1	9.0	0.015	0.012	−11.2
C	9½	18.6	49.30	46.54	46.98	5.861	5.544	5.642	339	282	5.6	4.7	0.025	0.021	− 9.6
	29½	17.5	50.05	45.68	46.99	6.130	5.351	5.643	534	363	8.8	6.0	0.014	0.009	− 6.9
D-1	9½	13.4	47.60	44.82	45.22	6.754	6.049	6.150	304	258	5.9	5.0	0.042	0.036	− 0.3
	29½	13.8	48.40	44.02	45.24	6.716	5.851	6.147	477	335	9.2	6.5	0.021	0.015	− 6.5
D-2	9½	11.4	46.15	43.80	44.14	6.531	6.361	6.447	270	230	5.1	4.3	0.045	0.038	−12.5
	29½	11.6	47.70	43.03	44.02	6.900	6.188	6.442	523	403	9.9	7.6	0.027	0.022	−14.4
E-1	9½	14.1	39.45	37.93	38.36	6.070	5.898	6.006	186	132	3.9	2.8	0.023	0.016	− 6.2
	29½	11.3	38.90	37.06	38.33	6.014	5.690	6.005	228	70	4.8	1.5	0.011	0.003	− 3.8
E-2	9½	12.7	38.15	36.54	36.87	6.230	5.966	6.051	208	164	4.2	3.3	0.029	0.023	− 4.7
	29½	10.8	37.25	35.77	36.75	6.007	5.799	6.050	197	65	4.0	1.3	0.010	0.003	− 5.2
F-1	9½	9.3	43.65	42.02	42.36	6.911	6.221	6.309	152	120	3.8	2.9	0.030	0.023	+ 5.8
	29½	10.7	43.70	41.33	42.36	6.818	6.052	6.313	223	123	5.4	3.0	0.012	0.007	+ 3.3
F-2	9½	12.2	41.70	40.28	40.67	6.446	6.180	6.280	130	104	3.1	2.5	0.019	0.015	− 1.7
	29½	10.4	41.33	39.44	40.58	6.470	5.986	6.278	194	75	4.6	1.8	0.011	0.004	− 0.1
									Mean at 9½ min.				0.031	0.025	
									S.D.				0.008	0.003	
									Mean at 29½ min.				0.014	0.009	
									S.D.				0.005	0.006	

* Cumulative average increase above resting.
† Values corrected for infused saline.
‡ Values uncorrected for infused saline.
§ Calculated from hematocrits.
‖ cs and ucs values are the same.

For the first nine and a half minutes the unit rate of filtration (URF), representing ml. filtered per Kg. body weight per cm. rise of venous pressure per minute, averaged 0.031 cs (0.025 ucs). The URF for 29½ minutes was always much smaller, averaging 0.014 cs (0.009 ucs). In most experiments the ratio of URF for 29½ minutes to URF for 9½ minutes was about 1:2 cs (1:3 ucs).

4. Serum proteins and optical density

During the period of Valsalva maneuvers, the fluid loss calculated from the change in serum protein concentration was always less than that calculated from hematocrits. This relationship became reversed, or at least the values approximated each other, during recovery. Examples of these directional changes are illustrated in Figure 2. There was a calculated loss of total circulating protein in all experiments except F-1. This loss appeared to have occurred chiefly during the first part of the period of Valsalva efforts, because the calculated loss at 29½ minutes was rarely more than that at 9½ minutes (see last column, Table III).

The observed changes in optical density provide only qualitative information because rates of disappearance of T-1824 during rest were not established. If it is assumed that the dye left the blood at an initial rate of 10 per cent per hour (12) and that the rate was unchanged during the procedure, the results are as shown in Figure 2. In the early part of the experiment the apparent volume filtered approximated that calculated from

the protein data, whereas toward the end of the half-hour of Valsalva efforts, it lay close to the volume calculated from the hematocrits. During the recovery period, however, the curve representing volume changes based on optical density fell below the hematocrit and protein curves, indicating that the rate of dye loss was slower during recovery than during the period of efforts.

DISCUSSION

1. Possible influence of factors other than fluid loss from the blood on venous hematocrit

Some of the limitations of calculating changes in plasma volume from hematocrits are shown by the results of the experiments with infusions of saline but without performance of Valsalva maneuvers. The differences sought between initial and observed values were necessarily so small that they could have been affected materially by spontaneous changes in the venous hematocrit. In 7 of the 10 experiments the blood samples taken before beginning the infusion showed evidence of the so-called "spontaneous hemodilution" which has been described repeatedly and which has been variously attributed to a gain of plasma volume or a rise of the body : venous hematocrit ratio (12–15). Thus, in six experiments the hematocrit of the last pre-infusion sample was 0.1 to 0.6 per cent [3] lower than that of the first, and in one (S. H., Table II), the fall amounted to 1.2 per cent. This was the experiment in which plasma volume appeared to have expanded even more than could be accounted for by the infusion, even at the time of the 30 minute sample. It seems probable, therefore, that the variability of the results of these experiments was caused chiefly by individual differences in the degree and persistence of spontaneous hematocrit changes following the initial venipuncture. In 8 of the 10 Valsalva experiments the hematocrit fell 0.2 to 0.7 per cent during the initial rest period. However, the minor fluctuations characteristic of the resting state probably did not continue during the Valsalva efforts when major circulatory derangements and rapid capillary filtration were in effect.

The addition of saline to the circulation had little influence on the results during the early part of the Valsalva experiments when the filtered volumes were large and infused volumes small. By the end of 30 minutes the influence of the saline was relatively more important, particularly in those experiments where the volumes of filtrate were small. The results of the infusion experiments where no Valsalva maneuvers were performed show that the "true" values probably were about midway between the results calculated as though all the saline remained intravascular (cs) and as though all of it had left the blood (ucs).

Three factors must be considered which might conceivably have caused systematic errors in estimating fluid movements from changes in the venous hematocrit under the conditions of these experiments. *1.* Cell-rich blood might have been transferred from some reservoir into the general circulation during the Valsalva efforts. There is no evidence to support this suspicion. Although about 200 ml. of blood can apparently be expressed from the lungs by a five second maximum Valsalva effort (16), this represents a redistribution of whole blood rather than a transfusion of cells because the hematocrit of the pulmonary circuit is about the same as that of the body as a whole (17) and the quantity of blood involved is only a small fraction of total blood volume. Finnerty, Buchholz and Guillaudeu (18) recently reviewed and contributed to the evidence against the presence, in healthy men, of storage areas which are capable of contributing cell-rich blood to the circulation. *2.* Theoretically, the reduced rate of blood flow in small vessels during a Valsalva maneuver would tend to lower the body : large vessel hematocrit ratio. Although this ratio tends to be deranged during severe circulatory disturbances (19) and may be reduced in heart failure as much as 2 per cent (20) to 4 per cent (21), our results were probably not affected in this way because the blood samples were taken during intervals of rest and not during Valsalva efforts. *3.* Cell volume might have been affected by changes in blood gas contents or pH caused by breath-holding and venous congestion. Landis and associates (2) have summarized this problem as it pertains to the effects of venous congestion. The good agreement which was found between calculated fluid losses based on hemoglobin and on hematocrit values by them (2) and by Henry, Hendrickson, Movitt and Meehan (8) indicates that with severe venous congestion or prolonged pressure breathing, sig-

[3] Hematocrit scale divisions.

nificant changes in cell size either do not occur or are reversed during aerobic handling of the blood samples *in vitro*. There is no reason to suppose the error from this source was any greater in our experiments. The samples were collected during intervals of rest, and they were freely exposed to air during transfers and while being mixed with anticoagulant before filling the Wintrobe tubes. Should any of the three sources of error just discussed be in effect, it would make the estimate of fluid loss based on hematocrits appear falsely high. Such systematic errors, at most, could not exceed the observed differences between the fluid losses calculated from hematocrits and those calculated from changes in protein concentrations. They could, however, account for some or all of the apparent loss of protein to the capillary filtrate.

2. Venous pressure and fluid movement during Valsalva maneuvers

The fidelity of the system used here to record venous pressure was probably adequate. Rushmer (22), using optical manometers, found that antecubital venous pressure rises slowly (0.74 mm. Hg per second) and reaches its maximum in 20 seconds during Valsalva maneuvers. Our curves have similar characteristics. Although pressure rises more rapidly in veins lying below the level of the heart than above (22), the changes observed in the antecubital vein when located just below heart level in the recumbent subject, as in our experiments, represent an approximate average for the body.

The average increase of antecubital venous pressure corresponding to a given percentage depletion of blood volume in our experiments was less than one-third of the airway pressure required to produce similar losses in humans (8) and in anesthetized cats (9) subjected to pressure breathing for like periods of time. This illustrates the fact that peripheral venous pressure may rise to only one-third or one-half of airway pressure during Valsalva efforts, the exact relationship apparently depending on the elasticity of the lung, the severity of cardiac tamponade, and the intensity of vasomotor responses (22, 23).

Landis and Gibbon (3), measuring changes in extravascular volume by pressure plethysmograph, found the URF during 10 minutes of venous congestion of the human forearm to be 0.0033 ml. per cm. of venous pressure per minute per 100 Gm. of tissue. Our values, expressed per 100 Gm. body weight, are similar but slightly smaller (0.0031 cs; 0.0025 ucs). Hamilton, Woodbury and Harper (24) found that effective capillary pressure fails to rise within thorax, abdomen and skull during cough and strain because intravascular and extravascular pressures rise equally. The URF for 9½ minutes of Valsalva efforts becomes 0.0036 cs (0.0029 ucs), in close agreement with the URF for 10 minutes of localized congestion of the forearm (3), if the contents of these protected cavities are taken to represent 15 per cent of body weight and the calculations therefore based on 85 rather than 100 per cent of body weight.

In contrast, the URF for 29½ minutes of Valsalva efforts was only one-third to one-half as great as the URF for 30 minutes of forearm congestion as found in three investigations: 0.0014 cs (0.009 ucs), as contrasted with 0.0023 (1), 0.0028 (3) and 0.0027 (4). It appears that a sustained rise of venous pressure ceases to be effective as a cause of capillary filtration much sooner when large portions of the body are involved than when congestion is localized. Thus, the URF decreased 50 per cent or more between 9½ and 29½ minutes of Valsalva efforts, but only 18 per cent between 10 and 30 minutes of forearm congestion (3).

The hemoconcentration resulting from Valsalva efforts is comparable in degree to that found when venous hypertension of like severity and duration is produced by other means, such as orthostasis (6, 7), thigh cuffs (5) or pressure breathing. The rapid decline of filtration after the first 10 to 20 minutes of extensive congestion has been noted by others (5, 6, 9).

An explanation for the more rapid cessation of filtration during generalized as compared with localized venous hypertension is to be found in the rapid rise of plasma oncotic pressure. Comparison of predicted with observed values (Table III) shows that the protein concentration of the plasma rose in some instances as much as 0.7 Gm. per cent after 9½ minutes and 0.9 Gm. per cent after 29½ minutes of Valsalva efforts. In the case of localized congestion, continued filtration is opposed only by rising tissue pressure, but when con-

gestion is widespread the influence of rising oncotic pressure is added, because relatively large amounts of protein-poor fluid leave the blood early in the course of the experiment and lymphatic return of protein is unimpaired. It seems unnecessary to invoke other explanations for the cessation of filtration after the early loss of 3 to 10 per cent of blood volume even though it is known that the mechanisms for conservation of body water may be set off by this degree of depletion (25, 26). Under the conditions of our experiments the basal rate of urine flow must have been small (27), so that even a 50 per cent reduction of flow (25, 26) could have had little influence on our results.

3. Protein and dye loss

The calculated losses of protein to the capillary filtrate deserve some consideration despite their relative unreliability because similar data are available in the literature for comparison (2, 8). Our results were fairly consistent, particularly in the first six experiments (Table III) where fluid movements were fairly large. This suggests that variations due to random errors in hematocrit and protein values were generally smaller than those in the extreme examples chosen to illustrate the possible range of technical error (see Appendix). Appreciable protein was lost during the Valsalva efforts, so that the calculated content of the filtrate at 29½ minutes was about 1 to 2 Gm. per 100 ml. Henry and co-workers (8) found similar losses during pressure breathing, whereas Landis and his associates found that the filtrate formed during forearm congestion at similar pressures was free of protein (2). Possible explanations for the apparent difference in protein loss during localized compared to more generalized congestion seem to be that *a*) during Valsalva maneuvers or sustained pressure breathing, effective capillary pressure might rise sufficiently in permeable areas such as the liver to cause loss of protein-rich fluid, or *b*) factors other than loss of fluid from the blood might contribute to the rise of hematocrit during the Valsalva maneuvers or pressure breathing, but not in localized congestion. Such factors might include changes in blood gas contents and pH or disturbances in the body: venous hematocrit ratio, as discussed earlier in this report.

Protein appeared to be re-entering the circulation, presumably via the lymphatic system, during recovery and possibly in the latter part of the Valsalva period, as evidenced by the increase in total circulating protein between 9½ and 29½ minutes seen in several experiments. Although Hyman and Goodman (9) found the rate of disappearance of T-1824 from the blood was unaffected by pressure breathing, our data show that dye loss was slightly more rapid during the period of Valsalva efforts than during recovery.

4. Clinical implications

The amounts of fluid leaving the blood during 10 to 30 minutes of repeated "bearing down" efforts are sufficient to account for the rise of hematocrit usually seen during the second stage of obstetric labor (28). Because the loss of fluid caused by venous hypertension is stopped by rising plasma oncotic and tissue pressures, the modest elevation of venous pressure caused by postpartum administration of oxytoxic drugs (10) would not be expected to have further effect, except to limit the rate of reabsorption of fluid filtered during labor. However, the same rise of venous pressure after cesarean section not preceded by labor would be expected to cause some hemoconcentration (10).

It would appear from our results that in heart failure, clinical edema could not result from venous hypertension without concomitant renal retention of salt and water, since massive capillary filtration is self-limited. However the results of the Valsalva experiments cannot necessarily be applied quantitatively to the problem of cardiac edema because factors influencing the rate of rise of interstitial and plasma oncotic pressures during capillary filtration may be different in the patient with edema compared to the healthy subject. In the patient the initial volume of fluid filtered per unit rise of central venous pressure might be greater than suggested by our results because the capillary beds of thorax and abdomen would be affected, whereas they were "protected" in the Valsalva experiments. The resultant depletion of plasma volume might call into play the homeostatic mechanisms regulating fluid balance more rapidly. On the other hand, plasma oncotic pressure might not be maintained as well in the cardiac patient as under experimental conditions because

a) protein-rich fluid would probably be filtered in the liver, and *b*) renal retention of salt and water would tend to dilute the plasma. Local factors to be considered are *a*) reduced tissue elasticity and *b*) impaired lymphatic drainage, as found by McMaster (29) in patients with cardiac edema. The former would cause tissue pressure to rise less rapidly and thus enhance filtration. The latter would work in the opposite direction. The possible effects of altered total return of fluid and protein via lymphatics to the blood cannot be estimated.

SUMMARY

1. In 10 experiments on healthy subjects, systemic venous pressure was raised 11 to 21 cm. H_2O for half-hour periods by repeated performance of Valsalva maneuvers. Changes in venous hematocrit, plasma protein and T-1824 concentrations showed that fluid left the blood rapidly during the first 10 to 20 minutes. Filtration stopped or diminished greatly after an initial loss of 3 to 10 per cent of blood volume.

2. During the first 10 minutes the unit rate of filtration was similar to that observed during localized congestion of the forearm, if it is assumed that there was no rise of effective capillary pressure and no filtration within thorax, abdomen or skull. Cessation of filtration was more abrupt than in the locally congested forearm. The extensive loss of protein-poor fluid and more rapid establishment of a new balance of forces across the capillary walls when venous congestion is generalized can account for the difference.

3. The apparent concentration of protein in the filtrate was about the same as that observed in experiments with pressure breathing, but greater than that noted during forearm congestion. Total circulating protein usually failed to decrease further after the modest loss which occurred during the first 10 minutes. The rate of loss of T-1824 from the blood was slightly more rapid during the period of Valsalva efforts than during recovery.

4. It is concluded that the quantity of fluid leaving the blood during 10 minutes of Valsalva efforts is sufficient to account for the hemoconcentration commonly associated with parturition. Because of its self-limited nature, capillary filtration cannot be solely responsible for the production of generalized clinical edema.

APPENDIX

Symbols and equations. The following procedures were used in calculations, where

H = hematocrit
P = plasma protein concentration
Vrbc, Vpl, and Vwb = total volumes of cells, plasma and blood
TP = total circulating protein (P × Vpl)
x_h = volume of fluid lost per 100 ml. of blood, calculated from hematocrits
$\bar{X}_h$ = total volume of fluid lost, calculated from hematocrits
x_p = volume of fluid lost per 100 ml. of plasma, calculated from protein concentrations
$\bar{X}_p$ = total volume of fluid lost, calculated from protein concentrations
$\bar{P}$ = loss of circulating protein
P_x = protein concentration of filtrate.
Subscripts o and p refer to observed and predicted values, respectively.
Equations:

1. $x_h = 100 - 100 \times \frac{H_p}{H_o}$
2. $\bar{X}_h = x_h \times Vwb$
3. $x_p = 100 - 100 \times \frac{P_p}{P_o}$
4. $\bar{X}_p = x_p \times Vpl$
5. $\bar{P} = TP_p - P_o \times (Vpl - \bar{X}_h)$
6. $P_x = \frac{\bar{P}}{x_h}$

Sample calculation. The handling of the data for nine and a half minutes, Experiment A-1 (line 1, Table III) may serve as an example. Vpl and Vwb for this experiment were calculated from the resting hematocrit, using the value for Vrbc which was obtained in the companion experiment on the same subject, A-2, in which plasma volume was measured.

Predicted values for nine and a half minutes were derived and tabulated as follows:

	Initial values	Losses from two preceding samples, and gains from infused saline	Predicted	
			cs	ucs
H (*ml. %*)	47.60		47.04	47.61
Vrbc (*ml.*)	2,785	− 7	2,778	2,778
Vpl (*ml.*)	3,065	− 7	3,127	3,058
Vwb (*ml.*)	5,850	−14	5,905	5,836
P (*Gm./100 ml.*)	5.990		5.851	5.989
TP (*Gm.*)	183.594	− 0.444	183.150	183.150
Saline (*ml.*)		+70		

Using the values corrected for infused saline (cs), Equations 1 to 6 give the following results:

1. $x_h = 100 - 100 \times \frac{47.04}{49.85} = 5.64$ ml./100 ml. blood
2. $\bar{X}_h = 5.64 \times 59.05 = 333.04$ ml.
3. $x_p = 100 - 100 \times \frac{5.851}{6.322} = 7.46$ ml./100 ml. plasma

4. $\bar{X}_p = 7.46 \times 31.27 = 233.3$ ml.
5. $\bar{P} = 183.149 - 6.322 \times (31.27 - 3.33) = 6.5$ Gm.
6. $P_x = \frac{6.5}{333} = 1.95$ Gm./100 ml.

Accuracy of calculated results. The accuracy of H_p and H_o affects the reliability, not only of x_h and $\bar{X}_h$, but of $\bar{P}$ and P_x. We have found the standard deviation of a single venous hematocrit to be 0.27 ml. per cent. This is based on measurements of three successive samples taken from an indwelling needle at five minute intervals in each of 103 experiments, and includes the effects of spontaneous fluctuations in the venous hematocrit, as well as analytical error when each hematocrit is measured in duplicate. In the experiments reported here, the most extreme error which might be anticipated from random deviations in H may be calculated by adding 0.27 to H_p and subtracting 0.27 from H_o. The values representing the largest and smallest filtration volumes of the series, *i.e.*, 29½ minutes, Experiment B, and 9½ minutes, Experiment F-2 (see Table III), may be used to test the effects of this manipulation. In the instance of the largest filtered volume, the apparent value of $\bar{X}$ is reduced 10 per cent (from 687 to 621 ml.), while $\bar{P}$ and P_x are reduced 35 and 28 per cent, respectively. In the case of the smallest filtration volume, similar manipulation of the data lowers $\bar{X}$ 28 per cent and $\bar{P}$ 94 per cent. (This changes $\bar{P}$ from an apparent loss of 1.7 to a gain of 1.3 Gm.)

If it is assumed that the serum protein concentrations may err by ±0.16 Gm. per 100 ml. (11), the significance of our calculated values may be tested by adding 0.16 to P_p and subtracting 0.16 from P_o. With the data for 29½ minutes, Experiment B, this substitution makes $\bar{P}$ increase 127 per cent, or from 11 to 25 Gm., while P_x also rises more than 100 per cent (from 1.6 to 3.6 Gm. per 100 ml.).

Henry, Goodman and Meehan (30) found that if the numerical value of the product $(H_o - H_p)(P_o - P_p)$ is about 6, P_x may vary over a range of plus or minus 2 Gm. per 100 ml. due to random errors of determining P and H. As the product becomes progressively smaller, the variability increases. The corresponding products for our data range between 0.3 and 5.3, showing that the degree of hemoconcentration achieved in the experiments was not great enough to permit accurate calculation of P_x.

Thus, values for $\bar{X}_h$ can be relied upon within limits ranging from plus or minus 10 per cent of the largest and plus or minus 30 per cent of the smallest values in our series. $\bar{P}$ and P_x are dependable only in the general range of plus or minus 100 to 200 per cent.

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THE EFFECT OF THIAMINE DEFICIENCY ON HUMAN ERYTHROCYTE METABOLISM[1, 2]

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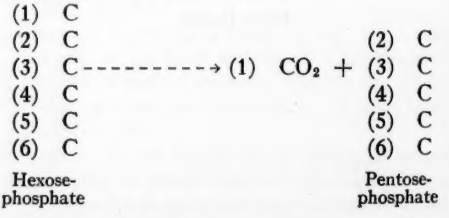
When mature mammalian erythrocytes are incubated in a glucose medium there is a low level of oxidation, but when methylene blue is added to the reaction mixture oxygen consumption and glucose utilization are greatly increased as a result of activation of the pentose phosphate pathway (1, 2). Non-nucleated erythrocytes possess the enzyme potential to recycle 5-carbon fragments (pentose) to glucose-6-phosphate but not to oxidize glucose-6-phosphate to pentose and carbon dioxide. Methylene blue, by virtue of its ability to carry electrons directly to oxygen, accelerates the mechanism for glucose oxidation, permitting the subsequent nonoxidative reactions of the pentose phosphate pathway to follow. Thiamine pyrophosphate is an essential cofactor for a reaction in this pathway (3, 4) and erythrocytes of thiamine-deprived rats, when incubated with methylene blue, have clearly exhibited impairment of the thiamine-dependent reaction (5-7).

In this report, observations have been extended to human thiamine deficiency. Studies from this laboratory have demonstrated that the opthalmoplegia of Wernicke's encephalopathy is not affected by bed rest, alcohol withdrawal and administration of a purified diet containing ascorbic acid and all of the B vitamins other than thiamine, but that when thiamine alone is added to the purified diet, rapid clearing of ophthalmoplegia ensues (8). It was felt, therefore, that patients with Wernicke's encephalopathy exhibiting extraocular muscle paralysis constituted a clearly defined example of human thiamine deficiency, and erythrocytes of nine patients manifesting this disorder were studied and compared with those from well-nourished controls and with those from a group thought to be deficient of B_1 but not exhibiting opthalmoplegia.

THEORETICAL CONSIDERATIONS

Provided the necessary substrate, enzymes and cofactors are present, the carbon compounds of the pentose phosphate pathway continuously recycle with regeneration of glucose-6-phosphate (9). Oxidation of glucose-6-phosphate and subsequent decarboxylation of 6-phospho-gluconate produces carbon dioxide arising from the first carbon and the residue is a pentose, the first carbon of which was the second carbon of the original glucose molecule:



Subsequent reactions are nonoxidative. Ribulose-5-phosphate, the pentose phosphate resulting from the oxidation of 6-phospho-gluconate, can be converted to xylulose-5-phosphate and ribose-5-phosphate by enzymes (epimerase, isomerase) that are widely prevalent in animal tissues (10). Pentose phosphate may then participate in the transketolase reaction where thiamine pyrophosphate acts as an essential cofactor (3, 4), and in

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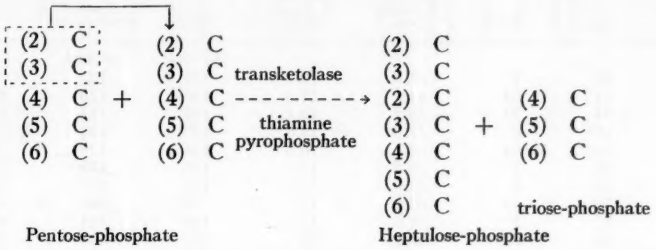
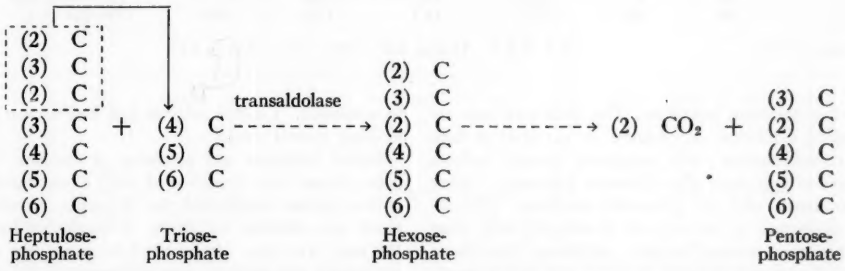
[3] Public Health Service Research Fellow of the National Heart Institute.

which the active glycolaldehyde portion of xylulose-5-phosphate condenses with ribulose-5-phosphate (11). The product is the seven carbon sugar, sedoheptulose-7-phosphate. The second carbon of the original glucose molecule is now the first carbon of the sedoheptulose:



In the transaldolase reaction (12) the first three carbon atoms of heptulose phosphate condense with the triose phosphate residue regenerating hexose phosphate. The first carbon atom of this new hexose molecule corresponds to the second carbon of the original glucose and CO_2 recovered from a second cyclic oxidation is derived from the original second carbon:



In accord with these considerations it has been found that when normal human erythrocytes are incubated with methylene blue and glucose labeled with radioactive carbon (C^{14}) in the first position, and, separately, with glucose labeled with C^{14} in the second position, the recoveries of carbon-1 carbon dioxide (C-1-$C^{14}O_2$) and C-2-$C^{14}O_2$ each fall within relatively narrow limits (Table I) (1). The first two carbon atoms of the glucose molecule are those principally involved in the oxidative process conforming to the observation that 95 per cent of radioactive carbon dioxide recovered when erythrocytes and methylene blue were incubated with uniformly labeled glucose could be accounted for by recoveries of radioactive CO_2 when glucose labeled only in the first and second positions was used as substrates (1).

Theoretically, thiamine deficiency would interfere with the cycle at the transketolase reaction. Oxidation of hexose phosphate would begin normally and the first carbon of the molecule would be recovered as C-1-$C^{14}O_2$ in the expected amount. Impairment of the transketolase reaction and subsequent lessened regeneration of new hexose phosphate, the first carbon of which would represent the original carbon-2, would result in diminished C-2-$C^{14}O_2$ recovery. As the reaction is altered after the decarboxylation of hexose, pentose would be expected to accumulate. Confirmation of these expected results has been achieved utilizing the erythrocytes of thiamine-deprived rats (5–7).

MATERIALS AND METHODS

Patients. All patients with suspected Wernicke's encephalopathy and/or beriberi brought to the authors' attention after their admission to the Boston City Hospital are included in this study.

Wernicke's encephalopathy (*Table II*). Nine subjects (seven male, two female) were studied. In eight, initial observations were made prior to thiamine administration and one (J.W.) was first studied three

TABLE I

C^{14} recovery, QO_2 and pentose accumulation in erythrocytes of 20 control subjects

Patient	Age	Sex	C-1 recovery % counts added	C-2 recovery % counts added	QO_2	Pentose accumulation	Diagnosis
					µl./3 hrs.	*µg./flask*	
W. B.	20	F	41.6	19.3	111	132	Normal
G. F.	41	M	41.2	21.3	125	120	Cirrhosis
J. B.	54	M	33.9	15.7	104	113	Cirrhosis
R. W.	19	F	37.6	18.0	123	121	Normal
C. D.	46	M	34.1	16.6	104	138	Normal
E. H.	36	F	39.5	13.1	106	158	Normal
D. M.	26	F	31.0	14.9	103	149	Normal
W. B.	55	M	32.6	12.7	97	109	Cardiac
C. B.	22	F	34.6	16.5	114	121	Enteritis
J. C.	46	M	40.1	16.6	100	144	Penicillin reaction
M. D.	41	F	40.7	20.2	112	118	Cirrhosis
J. M.	60	M	35.3	17.9	110	135	Cirrhosis
A. R.	67	M	36.3	17.4	98	135	Gastritis
M. W.	33	F	30.8	14.4	86	129	Normal
J. R.	54	M	34.5	13.9	102	135	Bronchiogenic carcinoma
L. B.	46	M	32.1	18.0	127	154	Pneumonia
S. W.	33	M	34.5	15.9	102	140	Normal
A. B.	36	F	31.5	16.6	102	133	Normal
A. S.	46	F	35.0	18.9	126		Thyrotoxicosis
J. S.	30	M	37.8	14.1	135	136	Normal
Mean ± S.D.			35.7 ± 3.5	16.6 ± 2.3	109 ± 12	133 ± 13	

hours after thiamine injection. The diagnosis was established by exhibition of weakness or paralysis of both lateral rectus muscles which completely cleared in from two hours to four days after thiamine treatment. Other signs consistent with the Wernicke syndrome (13) included nystagmus in seven (one developing only after return of lateral rectus function), peripheral neuritis in seven, confusion and delirium in eight, and ataxia in all. Four had smooth, red tongues. A diagnosis of portal cirrhosis of the alcoholic type was made on clinical grounds in three patients.

Suspected thiamine deficiency (*Table III*). Eight subjects (six male, two female) in this category, all alcoholics, were studied prior to thiamine administration. In five (E.B., J.B., A.K., F.C., C.A.) exhibiting ataxia, peripheral neuritis and confusion, Wernicke's encephalopathy was suspected but eliminated as ophthalmoplegia was absent. One (G.T.) had clinical scurvy, as well as nystagmus and peripheral neuritis. In two subjects with heart failure, edema and peripheral neuritis, beriberi was initially suspected, but was later discarded when other organic heart disease was recognized.

Control group (*Table I*). Twenty individuals (11 male, 9 female), 9 of whom were normal laboratory personnel and 11 hospital convalescents, were studied.

Diet evaluation. All patients with Wernicke's encephalopathy and suspected thiamine deficiency were chronic alcoholics and had been imbibing heavily before admission. Many had been found in rooms or on the street in states of inebriation and physical collapse. Diet histories were unreliable but it was presumed that all had failed to ingest solid food for at least one week prior to admission. Control subjects had had normal diets for at least several weeks.

Initial treatment and obtaining of samples. Intravenous glucose was administered until examination by one of the authors established the diagnosis, at which time blood was obtained for study. Thiamine hydrochloride, 100 mg., was then administered intravenously or intramuscularly and patients were subsequently fed according to clinical indications. Blood samples were obtained periodically after therapy as outlined in Table II, and clinical status ascertained by repeated physical examination. In one case of Wernicke's encephalopathy, thiamine had been given 3 hours before blood was obtained. Samples not immediately studied were refrigerated for a maximum of 8 hours. We have observed no loss of activity in either normal or deficient cells so stored for 12 hours or less.

Methods. Heparinized blood was centrifuged at 2,500 rpm for 20 minutes at room temperature. Plasma and buffy coat were removed and packed erythrocytes were diluted to approximately 50 per cent in phosphate saline buffer, pH 7.4 (NaCl, 0.015 M; KCl, 0.004 M; $MgCl_2$, 0.005 M; Na_2HPO_4–NaH_2PO_4, 0.02 M). Hematocrit was determined for each sample.

Standard Warburg technique was used. Into the outer chamber of 10 ml. Warburg flasks were placed 0.4 ml. prepared erythrocytes, 0.1 ml. 0.05 per cent methylene blue, 0.1 ml. 10 mg./ml. solution of C^{14}-labeled glucose,[4] and 0.4 ml. buffer; and into the center well, 0.2 ml. 15 per cent KOH. After equilibration for five minutes the

[4] C^{14}-labeled glucose obtained from H. S. Isbell, National Bureau of Standards, Washington, D. C.

TABLE II

C^{14} recovery, QO_2 and pentose accumulation in nine subjects with Wernicke's encephalopathy—Relation to therapy and clinical manifestations

Patient	Time	C-1 recovery % counts added	C-2 recovery % counts added	QO_2	Pentose accumulation	Abducens* paralysis	Nystagmus	Peripheral neuritis	Glossitis	Mental disturbance	Portal cirrhosis	Bilirubin	Complications
				μl./3 hrs.	*μg./flask*							*mg. %*	
E. W.	Pre-Rx	36.3	9.1	99	191	++	0	0	+	0	0	1.0	Beriberi
41	+15 hrs.	28.0	12.9	102	180	+	+						
Male	+ 7 days	29.9	10.7	107	161	0	+						
S. M.	Pre-Rx	34.9	11.0	101	179	+++	+	+	0	+	+	1.1	
53	+ 7 hrs.	29.1	12.4	106	167	+	+	+		+			Korsakoff psychosis
Male													
F. E.	Pre-Rx	31.0	9.5	74	145	++	+	0	+	+	0	2.4	
60	+ 8 hrs.	27.3	11.1	83	134	+	+			+	0		Persisting confusion
Male													
T. D.	Pre-Rx	38.4	11.3	96	147	++	0	+	0	+	0	1.3	Delirium tremens;
49	+ 9 hrs.	34.1	14.0	105	131	+	0			+			perforated peptic
Male	+33 hrs.	38.7	16.8	103	125	0	0			+			ulcer, operated;
	+ 6 days	33.8	13.7	103	123	0	0	+		+			Korsakoff psychosis
G. F.	Pre-Rx	39.6	13.6	101	186	+	+	+	+	+	0	0.9	
56	+ 6 days	36.3	14.0	100	216	0	+	+		+			
Female	+11 days	36.5	16.8	106	147	0	+	+		+			Korsakoff psychosis
R. S.	Pre-Rx	41.3	6.6	88	181	++	+	+	0	+	0	0.9	
52	+ 8 hrs.	31.4	12.9	108	205	0	0	+		+			Acute pancreatitis;
Female	+ 2 days	33.6	13.2	106		0	0	+		+			Korsakoff psychosis
W. B.	Pre-Rx	32.7	8.8	98	173	++	0	+	0	+	+	6.0	Hepatic coma
62	+18 hrs.	26.8	5.8	89	181	++	0	+		Coma			
Male	+ 4 days	31.7	14.9	118	137	+	0	+		+			Korsakoff psychosis
J. O.	Pre-Rx	39.1	11.7	129		+++	+	+	+	+	0	1.7	Delirium tremens;
53	+24 hrs.	28.3	12.1	119		0	+			+			Korsakoff psychosis
Male													
J. W.	+ 3 hrs.	37.7	12.5	98	144	+	+	+	0	+	+	4.8	
68	+14 hrs.	38.6	17.5	118	140	0	+			+			
Male	+2½ days	39.4	18.4	111	150	0	+	+		+			Persisting confusion

* Symbols indicate: weakness (+), palsy (++), and palsy and internal strabismus (+++).

TABLE III

C^{14} recovery, QO_2 and pentose accumulation in eight patients thought to be thiamine-deficient but not exhibiting ophthalmoplegia

Patient	C-1 recovery % counts added	C-2 recovery % counts added	QO_2	Pentose accumulation	Clinical state
			µl./3 hrs.	*µg./flask*	
E. B. 41 Female	32.5	15.2	108		Ataxia, nystagmus, peripheral neuritis, cirrhosis
J. B. 41 Male	37.1	16.7	101	162	Nystagmus, peripheral neuritis
G. T. 62 Male	33.5	14.8	107	143	Nystagmus, peripheral neuritis, scurvy
A. K. 58 Male	35.9	16.3	94	126	Confusion, ataxia, nystagmus, barbiturate intoxication
F. C. 54 Female	44.4	15.0	117	178	Nystagmus, ataxia, peripheral neuritis
C. A. 53 Male	33.7	13.6	85		Seizures, nystagmus, peripheral neuritis
W. D. 46 Male	36.5	14.7	107	144	Anasarca, CHF*, alcoholic Autopsy: acute bacterial endocarditis
A. R. 40 Male	36.6	16.1	130	122	Anasarca, CHF, cyanosis, alcoholic Final Dx: chronic cor pulmonale
Mean ± S.D.	36.3 ± 3.7	15.3 ± 1.0	106 ± 14	146 ± 21	

* Congestive heart failure.

mixture was incubated at 38° C. for three hours under air. The reaction was terminated by addition of 0.2 ml. 100 per cent trichloracetic acid and, after equilibration, radioactive CO_2 absorbed in the center well was precipitated as barium carbonate, plated and counted in a Robinson flow counter. The contents of the outer chamber were transferred and diluted with 10 per cent trichloracetic acid. Filtrates were analyzed for pentose by the orcinol method (14).

Duplicate samples were incubated separately with glucose-1-C^{14} and glucose-2-C^{14}, the specific activities of which had been determined by converting to osazones, plating and counting. Specific activity of glucose-1-C^{14} ranged between 4,100 and 17,000 cpm per mg. while that of glucose-2-C^{14} was between 5,500 and 27,000 cpm per mg. The fraction recovered as $C^{14}O_2$ of counts added initially is represented as C-1 and C-2, respectively, and is the arithmetical average of duplicate recoveries.

We have found a linear relationship between three-hour oxygen consumption and final hematocrit of prepared erythrocytes between 45 and 55 per cent, and figures for QO_2 were calculated from the arithmetical average of four corrected manometer readings (two incubated with glucose-1-C^{14} and two with glucose-2-C^{14}) further corrected to 50 per cent hematocrit.

Erythrocytes obtained before treatment from seven subjects with Wernicke's encephalopathy were incubated with thiamine hydrochloride [5] (0.25 mg.) and cocarboxylase [5] (0.1 mg.) to determine whether the metabolic defect could be corrected *in vitro*. Serum bilirubin concentrations were determined by the method of Ducci and Watson (15).

RESULTS

When erythrocytes of control subjects were incubated with labeled glucose and methylene blue, carbon-1 recovery ranged between 30.8 per cent and 41.6 per cent and carbon-2 between 12.7 per cent and 21.3 per cent. Minimum QO_2 was 86 µl. per three hours and maximum pentose accu-

[5] Purchased from Nutritional Biochemical Corp., Cleveland, Ohio.

TABLE IV

*C^{14} recoveries, QO_2 and pentose accumulation of various groups studied**

	C-1 recovery % counts added	C-2 recovery % counts added	QO_2	Pentose accumulation	Number of experiments
			µl./3 hrs.	*µg./flask*	
Controls	35.7 ± 3.5	16.6 ± 2.3	109 ± 12	133 ± 13†	20
Wernicke's encephalopathy, pretreatment	36.7 ± 3.6	10.2 ± 2.1 ($p = <0.01$)	98 ± 15	172 ± 18‡ ($p = <0.01$)	8
Wernicke's encephalopathy, maximum recovery after treatment	31.7 ± 4.3	13.8 ± 2.1	106 ± 11	148 ± 21‡	8
Wernicke's encephalopathy, pretreatment, thiamine added *in vitro*	35.5 ± 2.5	12.1 ± 1.9	107 ± 7	173 ± 35§	7
Wernicke's encephalopathy, pretreatment, cocarboxylase added *in vitro*	33.4 ± 3.8	11.9 ± 1.9	105 ± 8	178 ± 38§	7
"Suspected" thiamine deficiency	36.3 ± 3.7	15.3 ± 1.0	106 ± 14	146 ± 21§	8

* All figures are the mean plus or minus standard deviation.
† Represents 19 experiments.
‡ Represents 7 experiments.
§ Represents 6 experiments.

mulation was 158 µg. per flask (Tables I and IV). There appeared to be no relationship between various individual measurements and age, sex or physical condition of the donor.

Erythrocytes of patients with Wernicke's encephalopathy exhibited evidence of impairment of the transketolase reaction. In seven of eight experiments utilizing red cells obtained prior to therapy, carbon-2 recovery was less than the lowest control value. Mean carbon-2 recovery for this group was 10.2 per cent, significantly less than that of the control (16.6 per cent) (Table IV). In five of seven experiments pentose accumulation was increased and the group mean of 172 µg. per flask was significantly different from the control (133 µg. per flask). The extent of increased pentose accumulation did not correlate with that of depressed carbon-2 recovery. QO_2 was slightly diminished and carbon-1 recovery was not influenced. Erythrocytes of J. W., given thiamine two hours before blood was obtained, demonstrated depressed carbon-2 recovery. Observations made from 8 hours to 11 days after therapy revealed a return toward normal metabolic activity in terms of increased carbon-2 recovery, increased QO_2, and reduced pentose accumulation in the majority. A decrease in carbon-1 recovery was also noted (Table II and IV). In two instances (G. F., R. S.), however, an increased rather than a diminishing pentose accumulation parallel to increased carbon-2 recovery was observed. Patient W. B., suffering from severe liver disease and hepatic coma, did not have prompt improvement of ophthalmoplegia and his red cells obtained 18 hours after treatment did not demonstrate increased carbon-2 recovery. Four days later, recovery from hepatic coma and clearing eye signs were associated with return toward normal carbon-2 recovery. In all other subjects ophthalmoplegia cleared rapidly.

Other signs attributed to thiamine deficiency were slow in improving or failed to disappear. Of nine patients with Wernicke's encephalopathy, one achieved complete psychiatric recovery, two exhibited persistent confusion and disorientation at time of discharge, and six were transferred to mental hospitals with diagnoses of Korsakoff's psychosis. Nystagmus and peripheral neuritis also tended to persist.

Erythrocytes of eight subjects suspected of thiamine deficiency but not exhibiting opthalmoplegia had no impairment of the transketolase reaction as evidenced by carbon-2 recoveries, QO_2 and pentose accumulation in the normal range (Tables III and IV).

In seven experiments where deficient erythrocytes of patients with Wernicke's encephalopathy were incubated *in vitro* with thiamine and cocar-

boxylase, there was only slight increase in carbon-2 recovery and no change in pentose accumulation (Table IV).

DISCUSSION

An impairment of the transketolase reaction best explains the decreased recycling of carbon-1 of pentose, the carbon-2 of the original glucose molecule, to hexose phosphate and its subsequent recovery as $C^{14}O_2$ in methylene blue activated erythrocytes of patients with Wernicke's encephalopathy. As the first oxidative steps of the pathway were not influenced, recovery of C-1-$C^{14}O_2$ was not appreciably affected whereas that of C-2-$C^{14}O_2$ was significantly reduced. These findings are consistent with the thesis that dietary thiamine deficiency led to insufficient erythrocyte thiamine pyrophosphate as cotransketolase.

Following parenteral administration of thiamine to the deficient patients, a slow return toward normal metabolic activity as evidenced by increasing carbon-2 recovery was observed. Insufficient data are available to determine the time required for complete recovery. Experience with thiamine-deficient rats indicates this to be slow and incomplete (5, 6). Non-nucleated erythrocytes possess a limited capacity to phosphorylate thiamine compared with nucleated cells (16). This suggests that a new generation of erythrocytes must be produced before normal activity can be anticipated. Impaired phosphorlylation of thiamine has also been demonstrated in patients with hepatic cirrhosis (17) and the subject (W.B.) with the most severe liver disease exhibited the poorest response to therapy in terms of both regression of eye signs and improvement in erythrocyte metabolism. Delayed recovery of other coenzymatic functions of thiamine following treatment of patients with Wernicke's syndrome has recently been reported (18).

The failure of *in vitro* incubation of deficient human cells with cocarboxylase and thiamine to result in significantly increased carbon-2 recovery is in contrast to observations with rat erythrocytes in which definite albeit incomplete increased carbon-2 recovery was obtained (5–7). Species differences can be invoked since rat erythrocytes are known to have a greater capacity than human erythrocytes to phosphorylate thiamine to the active pyrophosphate (19). Cocarboxylase added *in vitro* is probably dephosphorylated before entering the erythrocyte (16, 17) and its action would be identical to *in vitro* thiamine. It has also been demonstrated that methylene blue, although stimulating erythrocyte respiration, does not stimulate thiamine pyrophosphate production and indeed may inhibit it (19), and possibly accounts for the failure of complete recovery in thiamine treated rat erythrocytes.

While evidence for reduced transketolase activity in thiamine-deficient erythrocytes is convincing from tracer studies, the supporting pentose data, although suggestive, are less clear cut. Both glucose remaining from the incubating medium and erythrocyte purine nucleosides and nucleotides that enter the trichloracetic acid filtrate give the orcinol reaction and tend to raise the apparent pentose concentratioin. This would tend to be greater in the deficient cells since less glucose is consumed. Moreover, decreases in pentose accumulation did not strictly parallel increased carbon-2 recovery in erythrocytes of treated patients.

Erythrocytes of patients known to have been imbibing heavily, eating poorly and exhibiting physical signs often ascribed to thiamine deficiency, but not suffering from ophthalmoplegia, exhibited no metabolic defect. Moreover, carbon-2 recovery correlated well with resolution of eye signs in the patients with ophthalmoplegia, and the single patient whose signs did not rapidly improve also exhibited persisting erythrocyte abnormality. It is probable that the red cell phenomenon occurs only in the most deficient subjects, *i.e.*, those with ophthalmoplegia, but consideration should be given to the possibility that the rapidly reversible aberration interfering with normal cranial nerve function is related to defective glucose catabolism with red cell and nerve cell being involved simultaneously. Available information indicates, however, that mammalian brain glucose catabolism is primarily by glycolytic pathways (20–22). Since nystagmus, ataxia and peripheral neuropathy have been clearly related to thiamine deficiency (8, 23–25), normal erythrocyte metabolism in patients with these signs but not ophthalmoplegia may be due to differences in severity of deficiency required before one or another coenzymatic function of thiamine is impaired. Furthermore, as slow disappearance of these signs is not unusual, the patients involved may have demonstrated stigmata of an

earlier episode of thiamine deficiency and may have been in a state of relative vitamin adequacy at the time they were studied.

Disturbances of carbohydrate intermediary metabolism recognized in thiamine-deficient states have provided a basis for biochemical evaluation of thiamine deficiency (26). Keto acid concentration in the blood is known to be increased in thiamine deficiency and has been used to indicate the presence of deficiency (27–30). However, these changes have been observed in many diseases apparently unrelated to thiamine deficiency, for example, multiple sclerosis (31), cirrhosis (32), and combined systems disease (33), both in the fasting state and in response to carbohydrate loads. Other investigations of thiamine adequacy have included direct measurement of the blood concentration of the vitamin which has not proved useful (34), and measurement of urinary thiamine excretions which provide only an index of tissue thiamine stores (35).

Application of methylene blue stimulated erythrocyte metabolism to clinical evaluation of thiamine status in man awaits collection and compilation of considerably more data. Defects noted in patients with unequivocal deficiency vary as to degree and are sometimes minimal in individual cases. However, the erythrocyte represents a most available tissue for the study of the thiamine-deficient state and may provide a basis for its clinical measurement.

SUMMARY

1. Erythrocytes of patients with Wernicke's encephalopathy, when incubated with methylene blue and C^{14}-labeled glucose, exhibited failure of the first carbon of pentose (the second carbon of the original glucose) to recycle to hexose and be recovered as radioactive CO_2, indicating impaired transketolation of pentose to heptulose.

2. The defect in transketolation was partially reversed after treatment of the patient with thiamine. *In vitro* addition of thiamine or cocarboxylase to deficient erythrocytes did not significantly alter the defect.

3. Erythrocytes of patients with physical signs usually attributed to thiamine deficiency but without ophthalmoplegia exhibited methylene blue stimulated glucose catabolism in no way different from that of well-nourished controls.

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EFFECT OF INOSINE ON RED CELL PRESERVATION [1]

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The addition of inosine, a purine nucleoside, to acid-citrate-dextrose (ACD) solution has been proposed as a means for prolonging the storage period of blood used for transfusion purposes (1). Inosine appears to affect the carbohydrate and phosphate metabolism of the erythrocyte and thereby improve the viability of stored red cells (1).

Since an increase in the outdating period of blood would be of great value, this study was undertaken by four different laboratories in order to compare standard acid-citrate-dextrose and acid-citrate-dextrose-inosine (ACDI) as blood preservatives. In 109 studies the comparison of ACD and ACDI was made by biochemical and hematologic tests done *in vitro* and the survival of transfused Cr^{51}-tagged red cells measured *in vivo*.

This report is a compilation of the results obtained in all four laboratories.

METHODS

For each study approximately 450 ml. of blood was collected from healthy human volunteers in either ACD or ACDI solution. If less than 450 ml. of blood was collected this is indicated in the tables and figures. Both preservative solutions contained 67.5 ml. ACD, NIH Formula A. For the ACDI solution, 1.8 Gm. of inosine [3] was added to the ACD solution before autoclaving. In 15 studies two different inosine preparations [4] were tested and Seitz filtration was used as the method of sterilization. Three types of containers were purchased. Glass bottles were supplied by Baxter Laboratories. Type F plastic packs with attached donor set were supplied by the Fenwall Laboratories. Type C plastic packs without an attached donor set were supplied by the Cutter Laboratories.

Following collection, the blood was stored at 4° C. for periods of 20 to 43 days. After various time intervals of *in vitro* storage, portions of the blood were tagged with Cr^{51} and transfused to the original donor or to appropriate recipients. The methods of Cr^{51} tagging have been previously described (2–5). From 30 to 87.3 μc. of Cr^{51} was used with specific activities of from 0.90 to 1.752 mc. per mg. The survival rate was determined. The method for estimating the recipient's red cell volume differed in each laboratory. Dr. Strumia's group estimated the blood volume by the Evans blue dye method (2). Dr. Gibson's and Dr. Finch's groups used P^{32} by methods already published by each independently (3, 4). Dr. Crosby's group used radioiodinated serum albumin (RISA®) (5). The method for estimating the red cell volume did not appear to affect the results and for the purpose of presentation the survival results from each group were averaged. The apparent half-time survival of the red cells (T/2) was calculated where possible.

In four studies, after 21 or 28 day storage periods, a sample was removed for tests *in vivo* and *in vitro* and the blood was immediately returned to the refrigerator. Following a further two week storage period, the same tests were repeated on the same blood and in the same subject. For these studies, one-half of the usual amount of Cr^{51} was used for each *in vivo* study.

In four studies an entire unit of whole blood was transfused. The Cr^{51} was added directly to the entire unit of blood. During the transfusion the pulse and blood pressure were measured every five minutes. The uric acid content of 24 hour urine samples was measured in two instances.

In 39 of the studies, *in vitro* tests were performed on the day of collection and again on the day of transfusion. These included determinations of: *1)* mean corpuscular hemoglobin concentration (MCHC), *2)* plasma Na, *3)* plasma K, and *4)* plasma hemoglobin. No attempt was made to correct the plasma K for the potassium contributed by the lysed erythrocytes. In 41 studies the following *in vitro* determinations were made on the day of transfusion: *1)* mean corpuscular volume, *2)* pH, *3)* lactic acid, *4)* dextrose, *5)* adenosine triphosphate, and *6)* percentage of hemolysis in 0.63 and 0.60 per cent NaCl.

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[3] Purchased from Schwartz Laboratories.

[4] One preparation obtained from Zellstoff Waldhof-Werke, Mannheim, Germany; one preparation obtained from Schwartz Laboratories.

TABLE I

Comparison of ACD-inosine and ACD as blood preservatives

Subject	Days storage	MCHC	Na	K	Plasma hb.	24 hr. survival	T/2
		%	*mEq./L.*	*mEq./L.*	*mg. %*	%	*days*
			Group I. ACDI in bottles				
1	0	33.5	170.5	3.9	33.6		
	21	32.0	148.7	33.3	65.0	95	24
2	0	31.4	164.1	4.0	3.8		
	21	32.2	153.2	25.0	60.5	92	31
3	0	32.0	172.0	4.1	20.5		
	28	32.5	143.0	31.8	140.0	71	28
4	0	31.6	172.0	3.8	11.0		
	28	32.2	140.0	30.9	110.0	75	27
5	0	31.5	174.2	4.4	16.2		
	35	33.0	137.5	33.8	90.0	65	20
6	0	31.5	174.2	4.1	13.0		
	35	32.4	142.5	36.3	165.0	99	26
7*	36					63	
8*	36					77	
9*	36					53	
10†	39					20	
11†	39					30	
12*	42					50	
13*	42					69	
14*	42					59	
15*	0	30.0	167.5	4.7	16.2		
	42	30.7	149.0	36.9	216.0	72	23
16	0	30.2	157.5	4.0	10.0		
	42	30.0	140.0	34.4	205.0	32	20
			Group II. ACD in bottles				
17	0	30.7	152.5	4.4	26.0		
	21	29.0	152.0	23.0	120.0	69	25
18	0	30.8	160.0	4.5	15.3		
	21	31.5	159.0	21.0	145.0	73	30
19	0	29.8	161.2	4.1	29.0		
	28	30.8	147.5	34.8	205.0	69	34
20	0	30.9	167.5	4.6	14.0		
	28	30.4	156.2	26.0	85.0	76	25
			Group III. ACD in F bags				
21	0	30.0	145.0	4.1	4.3		
	21	30.3	154.5	29.6	92.0	90	20
22	0	30.8	165.1	4.5	2.8		
	21	31.3	159.5	23.3	22.0	78	30
23	0	30.3	170.0	4.0	2.8		
	28	28.4	137.5	31.3	214.0	54	23
24	0	28.9	147.1	4.3	2.5		
	28	30.3	140.0	30.8	194.0	80	28

* Four hundred twenty-five ml. blood collected.
† Two hundred ml. blood collected.

TABLE II

Comparison of ACD-inosine and ACD as blood preservatives

Subject	Days storage	MCV	MCHC	pH	Lactic acid	Dextrose	ATP	Na	K	Plasma hb.	Hemolysis 0.63% NaCl	24-hr. survival	T/2
		cu. μ	*%*		*mg. %*	*mg. %*	*mg. %*	*mEq./L.*	*mEq./L.*	*mg. %*	*%*	*%*	*days*
						Group IV. ACDI in F bags							
25	0		32.1					161.2	3.9	13.0			
	21		30.9					143.0	31.5	61.5		92	36
26	0		30.6					160.0	4.2	6.4			
	21		32.6					132.0	33.0	21.9		97	30
27	21	86		6.70	134	311	9.38			12.0	4	87	24
28	21	89		6.75	162	389	8.94			14.0	3	82	31
29	28	89		6.53	166	314	9.27			42.0	14	76	21
30	28	89		6.67	214	333	7.44			19.0	5	76	25
31	0		31.0					165.0	3.9	14.5			
	28		33.2					125.2	43.8	37.0		83	25
32*	0		31.6					168.7	4.2	6.6			
	28		32.2					139.5	34.8	20.0			
	0		31.0					161.0	4.6	4.5			
	28		28.1					150.0	16.3	20.0		81	29
33	34	89		6.63	180	340	8.94				9	55	22
34	34	92		6.60	248	282	8.15			58.0	7	34	20
35	35		32.9	6.57			3.04			39.0	11†	57	
36	35		34.1	6.51			3.57			42.0	14†	63	
37	0		31.1					170.7	5.6	8.4			
	35		32.2					139.0	20.6	16.5		68	29
38	0		32.3					160.0	4.6	5.9			
	35		31.7					138.0	32.5	27.0		76	27
39	0		31.3					165.0	4.6	5.4			
	42		32.7					142.5	35.8	107.5		73	25
40	0		32.0					174.2	5.2	5.4			
	42		32.9					142.5	35.8	146.0		37	24
41	42		33.3	6.49						50.0	21†	48	
42	43		30.3	6.54			3.62			115.0	33†	65	
43	43	85		6.54	275	279	2.49				15	35	14
44	43	86		6.67	184	331	7.14			108.0	18	32	20
						Group V. ACDI in C bags							
45	20	92		6.71	113	296	7.97			23.0	5	95	22
46	21	90		6.62	131	350	10.28				1	96	24
47‡	0		29.5					163.0	3.6	5.6			
	21		32.8					155.0	28.1	32.0		77	29
48	0		31.2					166.1	3.8	2.1			
	21		31.9					151.0	31.0	40.0		86	21
	35		32.0					153.0	39.0	67.5		58	28

* Results of two separate bleedings.
† Hemolysis in 0.6 per cent NaCl.
‡ Five hundred ml. transfusion.

TABLE II—*Continued*

Subject	Days storage	MCV	MCHC	pH	Lactic acid	Dextrose	ATP	Na	K	Plasma hb.	Hemolysis 0.63% NaCl	24 hr. survival	T/2
		cu. μ	%		*mg. %*	*mg. %*	*mg. %*	*mEq./L.*	*mEq./L.*	*mg. %*	%	%	*days*
49	0		30.4					156.2	3.9	2.4			
	21		31.6					148.2	35.3	38.0		88	30
	35		32.3					143.7	40.0	58.0		79	12
50	26	90		6.70	197	279	6.57			118.0	8	72	16
51	27	90		6.62	195	288	5.03			54.0	8	81	30
52	0		31.4					165.1	3.6	4.5			
	28		34.1					149.0	35.5	55.0		78	27
	42		32.9					135.0	40.0	121.1		63	16
53	0		29.6					165.0	4.6	4.5			
	28		31.5					127.5	33.6	65.0		85	25
54	0		29.6					167.6	4.3	4.0			
	31		31.3					132.5	44.4	74.5		73	17
	42		32.3					130.0	45.0	80.5		58	15
55‡	0		29.8					158.0	3.8	4.9			
	35		31.2					132.2	40.4	156.0		86	22
56	0		32.0					159.0	4.3	9.4			
	35		32.0					133.5	36.3	69.5		56	26
57	35	90		6.62	137	307	6.12			61.0	3	67	35
58	35	87		6.78	144	282	7.99			51.0	9	71	22
59§	36											59	
60§	36											64	
61§	36											60	
62	41	89		6.58	179	279	6.08			113.0	20	44	15
63	42	91		6.68	174	315	7.16			113.0	17	41	18
64§	42											44	
65§	42											49	
66§	42											42	
67	0		29.8					162.1	4.1	4.3			
	42		31.0					141.0	39.5	68.0		59	21
68	0		31.1					157.1	4.2	8.5			
	42		31.5					139.1	34.5	118.0		71	25
69‡	0		29.3					159.0	4.0	4.5			
	42		30.4					135.5	41.9	92.0		72	22
					Group VI. ACD in C bags								
70‖	21	95		6.71	113	308	8.48			33.0	3	85	25
71	0		32.2					170.0	4.0	6.6			
	21		32.6					152.0	40.0	46.0		99	33
72	0		32.6					165.0	4.4	6.4			
	21		32.7					155.0	40.0	39.0		73	31

§ Four hundred twenty-five ml. blood.
‖ Average of 10 bloods.

TABLE II—*Continued*

Subject	Days storage	MCV	MCHC	pH	Lactic acid	Dextrose	ATP	Na	K	Plasma hb.	Hemolysis 0.63% NaCl	24 hr. survival	T/2
		cu. μ	%		*mg. %*	*mg. %*	*mg. %*	*mEq./L.*	*mEq./L.*	*mg. %*	%	%	*days*
73	0		28.0					176.0	4.4	6.6			
	28		32.7					157.5	16.3	41.0		68	26
74	0		29.4					167.0	4.1	5.3			
	28		29.4					157.5	13.8	64.0		55	26
75¶	28	96		6.70	123	288	4.89			52.0	3	70	
76	35	90		6.80	128	307	3.84			168.0	9	37	15
77	35	98		6.70	177	223	3.71			176.0	16	54	30
78	42	93		6.59	142	284	2.67			126.0	7	41	24
79	43	97		6.69	170	211	1.08			113.0	9	47	20

¶ Average of 7 bloods.

Lactic acid was determined by the method of Barker and Summerson (6). Adenosine-triphosphate was determined by a method which was an adaption of the procedures described by Fiske and Subbarow (7), Hawk, Oser and Summerson (8), Gomori (9) and Lohmann (10). The remaining *in vitro* studies were done by standard methods. Before transfusion, Gram-stained smears were examined for bacterial contamination. None was found.

RESULTS

The results of the studies *in vivo* and *in vitro* are summarized in Tables I and II.

Except for an elevated plasma hemoglobin in units collected by vacuum in glass bottles, both initially and on storage, there were no significant differences between the different types of containers. A fall in plasma Na concentration occurred during storage. Concurrently there was an increase in the plasma K. The plasma hemoglobin also increased and an increasing percentage of red cells were hemolyzed in 0.63 per cent NaCl. Variable results were obtained in the measurement of pH, lactic acid and dextrose. However, there was an increase in lactic acid content and a trend toward decreases in both pH and dextrose on storage. In 35 of 45 determinations the MCHC increased on storage indicating the loss of some water. Inosine did not retard these *in vitro* changes. However, when adenosine triphosphate (ATP) levels were measured, the blood preserved in inosine showed a higher amount of ATP than control bags not containing inosine (Table II).

In vivo there was little loss of red cells during the first 15 to 30 minutes after transfusion (Figure 1). There appeared to be a greater loss at 30 minutes of red cells stored in bottles but this was probably not significant. Up to 21 days' storage, there was no apparent difference *in vivo* between units collected in ACD or ACDI. The survival of the transfused cells at 24 hours is graphically portrayed in Figure 2. All units stored in ACD for 21 days showed at least 70 per cent survival at 24 hours. All units stored in ACDI for periods up to 28 days showed at least 70 per cent survival at 24 hours. However, when storage was continued to from 35 to 42 days the survival of these cells was quite variable. Six of 20 units stored 35 days in ACDI had better than 70 per cent survival at 24 hours. However, only four of 23 units stored 42 or 43 days had 70 per cent survival at 24 hours. Where duplicate studies were performed, the decreased 24 hour survival is readily apparent (Figure 2). The type of container in which the blood was stored did not affect these results.

In general, the viable cells survived well; however, the T/2 decreased as the length of storage increased (Tables I and II). Five of six units stored in ACD had a T/2 of at least 25 days when stored for 28 days. Eight of nine units stored in ACDI for 28 days had a T/2 of greater than 25 days. However, all 14 units stored 42 days in ACDI had a T/2 of less than 25 days.

In four studies, 450 ml. of blood stored in ACDI for 21 to 42 days was transfused. This did not affect the *in vivo* results (Tables I and II). The

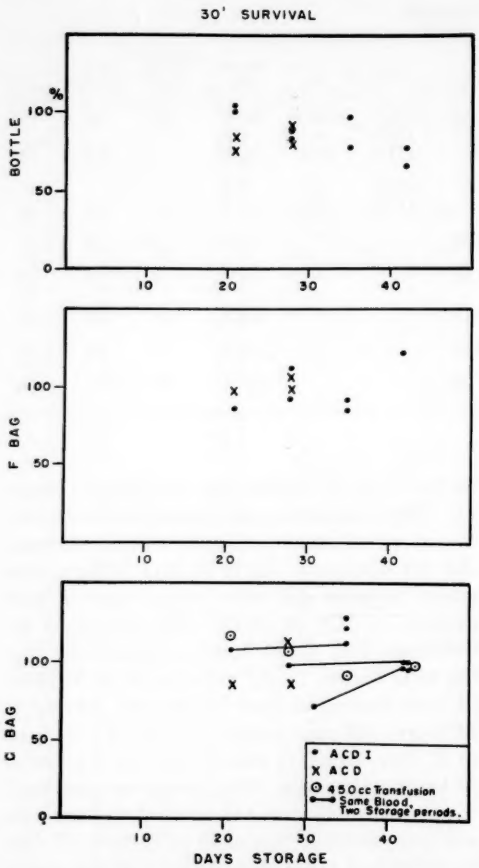

FIG. 1. COMPARISON OF 30 MINUTE SURVIVAL RESULTS IN DIFFERENT CONTAINERS, FOR VARYING PERIODS OF STORAGE USING ACD AND ACDI AS PRESERVATIVE SOLUTIONS

The percentage is based on the recovery of anticipated counts of radioactivity.

pulse and blood pressure were not affected by the transfusion. In two instances, the uric acid in a 24 hour collection of urine following the transfusion was found to be 1,072 and 1,940 mg. (normal values, up to 1,000 mg.).

The *in vitro* survival of red cells stored in two different inosine preparations [5] sterilized by Seitz filtration was studied. The results are summarized in Table III. With few exceptions the inosine employed was unable to effect satisfactory survival at either 36 or 42 days.

It was noted that in some of the specimens of blood stored with the addition of inosine, a rather heavy white precipitate appeared. Samples of this precipitate were tested by Dr. David R. Schwartz and found probably to consist of hypoxanthine. The hypoxanthine content was determined by direct ultraviolet measurement of the precipitate and confirmed by chromatography and spectrophotometric assay of the only ultraviolet absorping spot which appeared on the developed chromatogram.

DISCUSSION

The action of added inosine in blood preservation has recently been discussed by Gabrio, Donohue, Huennekens and Finch (1) and will not be dealt with in this report.

Gabrio and associates found the average survival of three units of blood studied by both Cr^{51} and Ashby techniques to be 82 per cent after *in vitro* storage of five and six weeks in ACDI. While similar results were obtained in 19 per cent of the studies reported here, there were, in general, poor survival results when the storage period was lengthened beyond 28 days. However, inosine did appear able to prolong the storage period for at least seven days.

There were certain technical differences between Gabrio's original study and the studies reported here. In the original study 120 ml. of ACD, NIH Formula B, was used. In the present study 67.5 ml. of ACD, NIH Formula A, was used. Formula B is composed of: trisodium citrate, 1.32 Gm.; citric acid, 0.48 Gm.; dextrose, 1.47 Gm.; with sufficient water to make 100 ml. Formula A is composed of: trisodium citrate, 2.2 Gm.; citric acid, 0.8 Gm.; dextrose, 2.45 Gm.; and sufficient water to make 100 ml. Both ACD solutions have been found to give adequate blood preservation for 21 days. With the addition of inosine, however, the effect of the different ACD preservatives used is unknown. In the original study the preservative was sterilized by scintered glass filtration and the inosine was added to the blood within 24 hours after it had been drawn into ACD. In the present study the inosine was added to the ACD and the preservative autoclaved. The

[5] One preparation obtained from Zellstoff Waldhof-Werke, Mannheim, Germany; one preparation obtained from Schwartz laboratories.

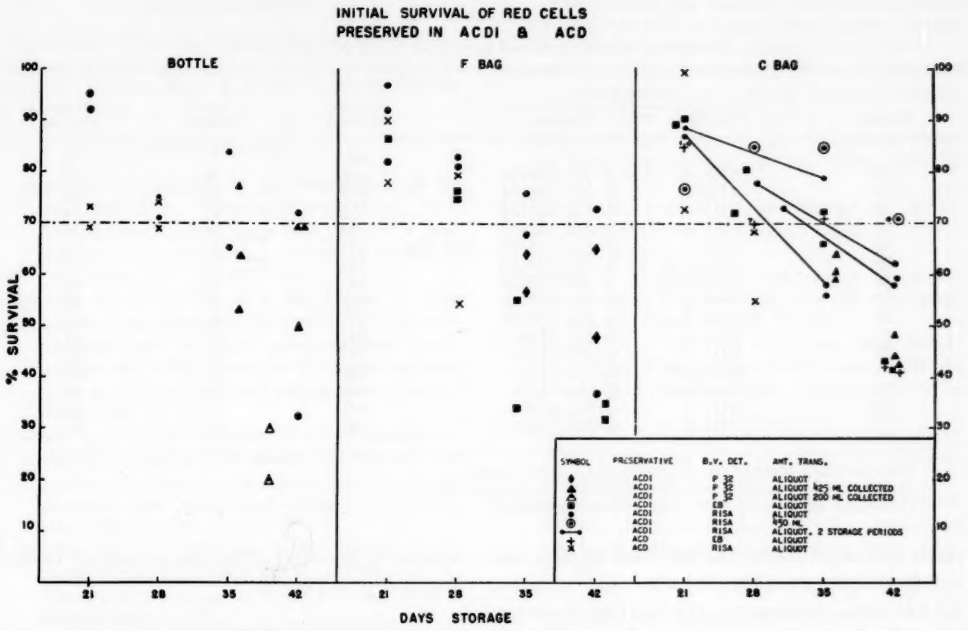

FIG. 2. COMPARISON OF AVERAGE 24 HOUR SURVIVAL RESULTS IN DIFFERENT CONTAINERS FOR VARYING PERIODS OF STORAGE USING ACD AND ACDI AS PRESERVATIVE SOLUTIONS

manufacturers have stated that inosine can withstand autoclaving. The inosine in both studies was supplied by Schwartz laboratories. Ultraviolet analysis of the inosine used in the present study revealed that the absorption ratios at various wave lengths and the E max were not significantly different from the other inosine preparations made by the manufacturers. The material was submitted to spectrographic analysis and no significant contamination by trace metals was found. The possibility remains, however, that there might be some difference in the inosine preparations. However, as reported in this paper, two different inosine preparations were tested after sterilizing the preservative solution by Seitz filtration. With few exceptions the inosine was unable to effect satisfactory survival at either 36 or 42 days.

Other than the increased number of studies in the present report, the difference in survival results obtained by Gabrio and co-workers (1) remains unexplained. Since some of the samples of blood stored in ACDI in the present study did give satisfactory survival results at 35 and 42 days, it has been suggested that red cells of different individuals may vary in their ability to withstand prolonged storage. Prankerd, in studying the effect of adenosine on the postincubation ability of red cells to utilize glucose, reaccumulate K + ion and resynthesize phosphate esters after storage, found considerable variability in the response of different blood samples. In fact, many samples failed to revive these metabolic functions at all (11).

The T/2 studies are of interest. The normal range for the T/2 of unstored blood is 25 to 35 days when Cr^{51} is used as a tag. For up to 28 days' storage the T/2 of ACDI blood was within normal range. On storage for 35 and 42 days in ACDI, the T/2 was shortened and this is further evidence of cellular damage on storage.

When the 30 minute survival of transfused red cells was measured, the units collected in glass bottles appeared to show a greater loss of cells than those units collected in plastic packs. This finding is of interest but is probably within the limits of experimental error since the percentage of

TABLE III

Survival of red cells preserved in various ACDI preparations * †*

Subject	Preservative			Volume of blood	Days storage	24 hr. survival
	ACD	Inosine				
				ml.		%
80	NIH–A	S. No. 105504		425	38	32
81	NIH–A	S. No. 105504		425	42	34
82	NIH–A	S. No. 105504		425	42	34
83	NIH–A	S. No. 105610		200	36	76
84	NIH–A	S. No. 105610	(0.82 Gm.)	200	36	70
85	Modified‡	S. No. 105610		200	36	70
86	Modified‡	S. No. 105610		200	42	46
87	NIH–A	S. No. 105610		200	42	68
88	NIH–A	S. No. 105610		200	42	61
89	NIH–A	G. No. 14701		200	36	67
90	NIH–A	G. No. 14701		200	36	53
91	Modified‡	G. No. 14701		200	36	55
92	Modified‡	G. No. 14701		200	42	48
93	NIH–A	G. No. 14701		200	42	49
94	NIH–A	G. No. 14701		200	42	55

* One and eight-tenths Gm. of inosine per 450 ml. blood.
† Blood stored in bottles; inosine sterilized by Seitz filtration.
‡ Citric acid reduced by half; total citrate unchanged.

viable cells at 24 hours was the same whether collected in bottles or in plastic packs. One observer did find some difference in the survival characteristics of cells stored in two types of packs but this experience was not borne out by the other investigators. One of the investigators also experienced difficulty in the collection of blood in plastic packs without the attached donor set. This difficulty also was not found by the other observers.

Other purine nucleosides have been tested by different techniques for their effect in prolonging the storage period of blood used for transfusion purposes (12, 13). Adenosine, one of these nucleosides, prolonged the storage period, but when injected intravenously caused an acute fall in blood pressure (6). This hypotensive effect was not shown by inosine or guanosine which seem able to some extent to improve the storage characteristics of refrigerated red cells (12, 13). The purine nucleosides are, however, metabolized to uric acid. While in healthy volunteers the transfusion of 450 ml. of blood was without apparent toxicity, the effect of multiple transfusions is not known. The possible toxicity of these purine compounds is under investigation at this time.

These studies have shown that the storage period of refrigerated blood could be extended to 28 days by the addition of 1.8 Gm. of inosine to ACD, NIH Formula A preservative solution. It is also hoped that the results will stimulate further interest in the effect of inosine on red cell metabolism and preservation.

SUMMARY

1. Acid-citrate-dextrose (ACD), NIH Formula A solution, with 1.8 Gm. of added inosine was studied as a blood preservative.

2. It was compared to ACD solution by *in vitro* tests and by the survival of Cr^{51} tagged red cells *in vivo*.

3. Inosine did not retard the *in vitro* changes in plasma Na, K, hemoglobin, lactic acid, dextrose or pH which occurred on storage. The changes in mean corpuscular hemoglobin concentration (MCHC) and percentage of cells hemolyzed in 0.63 per cent NaCl were not affected by the addition of inosine to the preservative solution. However, blood stored in inosine did show an increased amount of adenosine triphosphate when compared to blood preserved in ACD alone.

4. Using the criteria of 70 per cent survival of red cells 24 hours post-transfusion, blood may be stored in ACD for 21 days. In ACD to which inosine has been added (ACDI), blood may be stored for 28 days.

5. Slightly elevated urinary uric acid levels were found in two subjects after the transfusion of 450 ml. of blood. The possible toxic effects of multiple transfusions are not known.

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THE SERUM TRIHYDROXY–DIHYDROXY BILE ACID RATIO IN LIVER AND BILIARY TRACT DISEASE [1]

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Measurement of serum bile acid concentrations has long been expected to provide important information concerning hepatic and biliary tract disease because the formation and metabolism of these compounds is so closely associated with liver cell function. Contrary to expectations, however, serum bile acid determinations (chiefly cholic acid) have been of little value in the study of hepato-biliary disorders. After a careful study of serum cholates in liver disease, the conclusions of Sherlock and Walshe amply confirm this point (1). General experience, moreover, as listed in Table I, shows that there has been no uniform agreement concerning either the quantity or nature of the bile acids alleged to be present in normal serum. These shortcomings, rather than difficulties in quantitation, probably explain why serum bile acid determinations have not gained wider acceptance as a laboratory aid in the study of jaundice and liver disease.

In order to investigate these discrepancies, studies were done in this laboratory which showed that bile acids are demonstrable by paper chromatography in most normal human sera and that these acids are the same as those in bile and occur in approximately the same relative proportions (2, 3). A method for the simultaneous quantitation of the two major bile acid types was then devised and is described in the following sections. We found, as had previous investigators, that the serum concentrations of either the trihydroxy or dihydroxy bile acids were commonly elevated in patients with hepatobiliary disease, and although this information was not especially helpful, it soon became apparent that the ratio of the two main bile acid types often separated the patients into two groups. Those with predominantly hepatocellular injury had a trihydroxy-dihydroxy ratio of less than one, whereas those with obstructive jaundice usually had a ratio of greater than one. The significance of this ratio was briefly described in an earlier communication (4) and has been confirmed in a recent study by Rudman and Kendall (5). These investigators separated trihydroxy and dihydroxy acids by column chromatography and found that the dihydroxy acids predominated in the sera of patients with Laennec's cirrhosis, whereas patients with obstructive jaundice tended to have higher concentrations of trihydroxy acids. Schiff (6) has reported similar results employing the method described in this paper.

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METHODS

Certain features of the serum extractions described by Josephson (7) and Minibeck (8) were incorporated into a method employing alkaline hydrolysis. Briefly, this consists of precipitating serum proteins with alcohol and barium, separation of fats and neutral sterols from bile acids by partitioning between equal volumes of petroleum ether, ether, ethanol and water, and alkaline hydrolysis of the bile acids in the aqueous phase. The quantities of unconjugated trihydroxy and dihydroxy bile acids thus obtained were measured by ultraviolet spectrophotometry using the conditions described by Mosbach, Kalinsky, Halpern and Kendall (9) which permit a simultaneous differential analysis of these two bile acid types.

Preparation of reagents. The barium solution was prepared by adding 25 Gm. of anhydrous $Ba(OH)_2$ to 400 ml. of distilled water. The solution was heated, filtered and then 4 Gm. of anhydrous barium acetate was added. After cooling, the saturated solution is stable for months. The concentration of the sodium hydroxide solution was 8 per cent by weight in water. The 65 per cent sulfuric acid solution was prepared by combining 169 ml. reagent grade concentrated sulfuric acid (95 to 98 per cent) with distilled water in a 250 ml. volumetric flask. The petroleum ether used was Skelly Solve B (boiling point 60 to 68). It was found that peroxides greatly distort the bile acid absorption spectrum and hence it is important that the ether be peroxide free.

Procedure. One ml. of barium solution was added to 26 ml. of 95 per cent ethanol in a 50 ml. centrifuge tube marked at 30 ml. (Tube A, Figure 1). To this mixture, 3 ml. of serum was added drop by drop while stirring vigorously. The mixture was boiled for two minutes and

TABLE I

Normal serum bile acid concentrations obtained by different methods

Author	Date	Concentration	Bile acids
		mg. %	
1. Moleschott	1847	+	not specified
2. Hoover	1916	+	not specified
3. Rosenthal	1926	0	not specified
4. Perlzweig	1926	0	cholic
5. Coquellet	1927	4.28	sodium glycocholate
6. Aldrich	1928	3.2–5.1	glycocholic acid
7. Shattuck	1928	7–12	cholic acid
8. Gregory	1929	0	cholic acid (ox blood)
9. Charlet	1929	3.4–4.5	cholic acid
10. Tashiro	1931	0	cholic
11. Scheinfinkel	1933	0.8	cholic (dog)
12. Kusui	1933	8.8	cholic
13. Lichtman	1934	0.5–4.0	sodium glycocholate
14. Scott	1934	0	cholic
15. Chabrol	1934	0	deoxycholic
		0	cholic
16. Josephson	1935	1.6	cholic
17. Abe	1937	0	cholic } dog
		0	deoxycholic }
18. Minibeck	1938	0.25	cholic
19. Douglas-Saureman	1939	0	cholic
		0	deoxycholic
20. Jenke	1939	0	cholic
21. Gigon	1940	1	cholic
22. Thannhauser	1941	0.5–1.5	cholic (Jenke's method)
23. Irvin	1944	0.4–0.5	cholic
24. Sherlock	1948	1.5	cholic (Josephson method)
25. Kier	1952	0	cholic
		25–52	deoxycholic
26. Friedman	1952	5.3	cholic
27. Sobel	1953	2.1–2.5	cholic
28. Joppich	1953	0	cholic (children)
29. Wysocki	1955	0.86	cholic (Irwin method)
		0.35	deoxycholic (Szalkowski)
30. Rudman	1957	0	trihydroxy
		0	dihydroxy
31. Metais	1957	4.0	total bile acids

cooled in ice water. After centrifugation for 20 minutes at 3,000 rpm, the volume was adjusted to 30 ml. with ethanol and 25 ml. of the clear supernate was removed to a second tube (Tube B, Figure 1) and evaporated with reduced pressure in a tube heater. The residue was dissolved in 10 ml. of 50 per cent ethanol which had been previously equilibrated with equal volumes (25 ml.) of ethyl ether and petroleum ether. The pH was adjusted to less than 3.5 (Congo red paper) by the addition of about 0.1 ml. concentrated HCl. This solution was then extracted with the petroleum ether-ethyl ether phase which had been used for the equilibration. The ether phase was extracted with fresh, equilibrated 50 per cent ethanol and the combined aqueous ethanol phases were brought to a neutral pH with 8 per cent NaOH and then evaporated with reduced pressure in the tube heater to near dryness. Care was taken to avoid complete dryness, as baking makes the residue difficult to dissolve. To the residue from the aqueous phase, 5 ml. of 8 per cent NaOH was added, stirred to assure complete solution and placed in a pressure cooker at 15 pounds pressure for three hours. The resulting hydrolysate was then diluted with 5 ml. of distilled water, the pH was adjusted with concentrated HCl to less than 3.5 (Congo red paper) and extracted four times with 20 ml. volumes of ethyl ether. The ether phase was collected in a clean dry tube (Tube C, Figure 1) and after washing with water, was evaporated to complete dryness. To this residue, 5 ml. of 65 per cent sulfuric acid was added and the tube was immediately heated at 60° for exactly 15 minutes. After 10 minutes of cooling in ice water, the acid solution was filtered through a 2 cm. medium sintered glass funnel. The filtrate was poured into a quartz cuvette and the absorption spectrum determined at 10 mμ intervals from 220 to 410 mμ against 65 per cent sulfuric acid which was also heated at 60 degrees for 15 minutes. A Beckman DU spectrophotometer with hydrogen lamp and blue phototube was used.

Calculations. The observed optical densities were corrected for background absorption to obtain the true optical density using calculations similar to those described by Brice and Swain (10). To make the correction a constant was calculated from pure bile acid solutions which is independent of concentration and hence can be used to determine true optical density when the background absorption

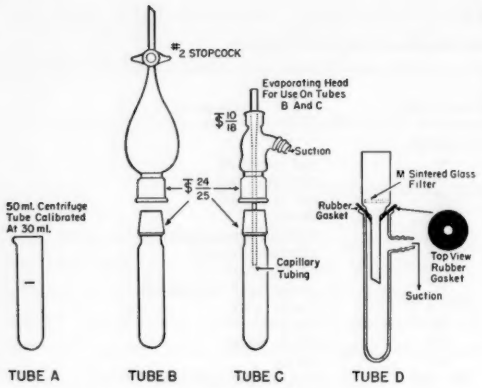

FIG. 1. GLASSWARE USED FOR SERUM BILE ACID DETERMINATIONS

of the solution and the concentration of the bile acids is unknown. Such a constant relates the optical density at maximum absorption to the average of two equidistant optical densities. Thus from the absorption spectra of 175 μg. samples of pure cholic and chenodeoxycholic acids dissolved in 65 per cent H_2SO_4 and heated for 15 minutes at 60° (Figure 2) the optical density at the wave length of maximum absorption for cholic acid was $E^0_{320} = 1.24$ and the values 20 mμ on either side of the maximum were $E^0_{300} = 0.775$ and $E^0_{340} = 0.215$. For chenodeoxycholic acid the values were $E^0_{380} = 1.78$, $E^0_{360} = 1.20$ and $E^0_{400} = 0.185$. The constant for cholic acid which relates the maximum optical density at 320 mμ to the average of two equidistant optical densities was obtained from the expression

$$\frac{E^0_{320}}{E^0_{320} - 1/2(E^0_{300} + E^0_{340})} = \frac{1.24}{1.24 - 1/2(0.775 + 0.215)} = 1.67;$$

the constant for chenodeoxycholic acid was similarly computed,

$$\frac{E^0_{380}}{E^0_{380} - 1/2(E^0_{360} + E^0_{400})} = \frac{1.78}{1.78 - 1/2(1.20 + 0.185)} = 1.63.$$

With the constants established, the true optical density of cholic or chenodeoxycholic acid in an unknown can be calculated by obtaining the difference between the optical density at their absorption maxima and the average of the adjacent optical densities, and multiplying by the constant. For cholic acid the equation is

$$E_{320} = 1.67[E^0_{320} - 1/2(E^0_{300} + E^0_{340})]$$

and for chenodeoxycholic acid the equation is

$$E_{380} = 1.63[E^0_{380} = 1/2(E^0_{360} + E^0_{400})].$$

Having thus corrected the observed optical densities for background absorption, the true optical densities were used for calculating the bile acid concentrations according to the following equations:

$$\frac{E_{320} \cdot K^D_{380} - E_{380} \cdot K^D_{320}}{K^D_{380} \cdot K^T_{320} - K^T_{380} \cdot K^D_{320}} = \text{Concentration of trihydroxy acids}$$

and

$$\frac{E_{380} \cdot K^T_{320} - E_{320} \cdot K^T_{280}}{K^D_{380} \cdot K^T_{320} - K^T_{380} \cdot K^D_{320}} = \text{Concentration of dihydroxy acids.}$$

The values for K were obtained by plotting known concentrations of pure bile acids against the optical density at 320 and 380 mμ (Figure 3), and are as follows:

K^T_{320} = optical density at 320 mμ per mg. cholic acid = 37.4

K^D_{320} = optical density at 320 mμ per mg. chenodeoxycholic acid = 8.06

K^T_{380} = optical density at 380 mμ per mg. cholic acid = 4.54

K^D_{380} = optical density at 380 mμ per mg. chenodeoxycholic acid = 51.3

Using these values in the preceding equations, the formulas for calculating the serum bile acid concentrations in μg. per ml. are obtained.

$(E_{320} \times 51.3 - E_{380} \times 8.06) \times 1.06 = \mu$g. of trihydroxy bile acid per ml. of serum

$(E_{380} \times 37.4 - E_{320} \times 4.54) \times 1.06 = \mu$g. of dihydroxy bile acid per ml. of serum

The factor, 1.06, is the quotient of $\frac{1{,}000 \times 5 \times 30/25 \times 1/3}{1{,}883}$

in which the numerator contains dilution constants and conversion factors which are apparent when the method is

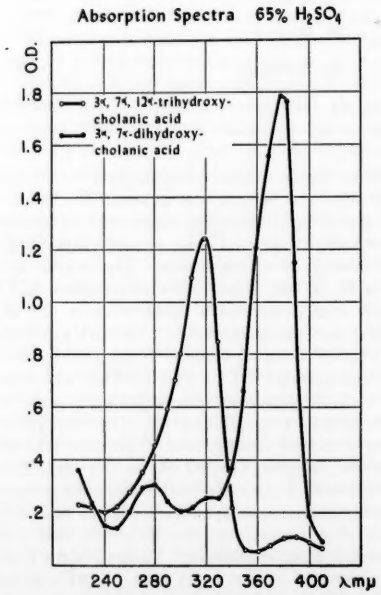

FIG. 2. ULTRAVIOLET ABSORPTION SPECTRA OF PURE BILE ACIDS IN 65 PER CENT SULFURIC ACID

examined and the denominator is the numerical value of $K^{D}_{380} \cdot K^{T}_{320} - K^{T}_{380} \cdot K^{D}_{320}$.

In correcting for background absorption, it was necessary to assume that this absorption changes linearly between the wave lengths used. This assumption is probably not strictly correct but approximates the facts closely enough to provide a more accurate answer than would result if this correction were not made. The amount of deoxycholic acid in normal human serum is quite small, when compared with chenodeoxycholic acid, as judged from paper chromatograms, and for this reason the absorption maximum for chenodeoxycholic acid (380 mμ) was used for the calculations. The absorption maximum for deoxycholic acid is at 385 mμ. The optical density at this wave length, however, is only slightly greater than at 380 mμ; consequently the error involved is slight and does not contribute significantly to the total error of the method. Plotting the spectrum aids in detecting any erroneous optical density values caused by a disproportionately high background or inexplicable absorption peaks.

Standard glassware is used except that two of the four tubes (Tubes B and C, Figure 1) have ground glass tops which fit the separatory funnel. This simple modification reduces the amount of glassware needed for the procedure and greatly improves recoveries. It also permits the use of a convenient evaporating head consisting of an arm for attaching a suction line and a capillary tube (Figure 1).

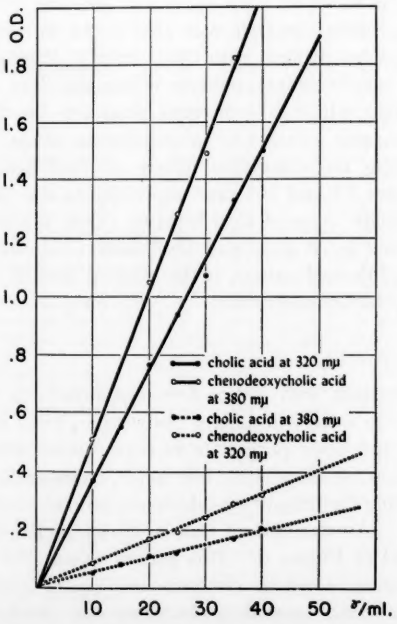

FIG. 3. OPTICAL DENSITY OF CHOLIC ACID AND CHENODEOXYCHOLIC ACID

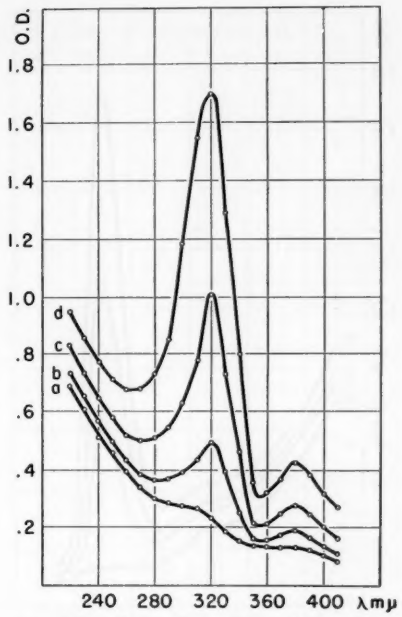

FIG. 4. ADDITION OF GLYCOCHOLIC ACID TO NORMAL SERUM

Amounts added per ml. of serum: *a*) none, *b*) 0.015 mg., *c*) 0.040 mg., *d*) 0.080 mg.

Experimental

The addition of dihydroxy or trihydroxy bile acids to serum is paralleled by an increase in the appropriate optical density value as shown in Figures 4 and 5. In these recovery experiments 15 to 100 μg. samples were used. Sodium glycocholate in water was added to the serum and chenodeoxycholic acid added to the alcoholic extract after partitioning with ether-petroleum ether. Results ranged from 74 to 91 per cent, mean 80 per cent. When solutions of pure bile acids were used in place of serum, recoveries were uniformly 100 plus or minus 5 per cent. Obviously there are a number of factors in serum which interfere with complete recoveries, and this limitation should be recognized when results are being compared and interpreted.

Duplicate determinations and normal values. In order to calculate the error of the method and the range of values to be expected in healthy subjects, duplicate determinations were done on single serum samples from 30 healthy persons ranging in age from 22 to 76 years, half of whom were women.

The average trihydroxy cholanic acid value was 1.4 μg. per ml. with an estimated standard deviation of 0.8 μg. per ml. and an estimated error of 0.8 μg. per ml. The average dihydroxy cholanic acid value was 0.76 μg. per ml. with an estimated standard deviation of 1.4 μg. per ml. and an estimated error of 0.36 μg. per ml. The normal values

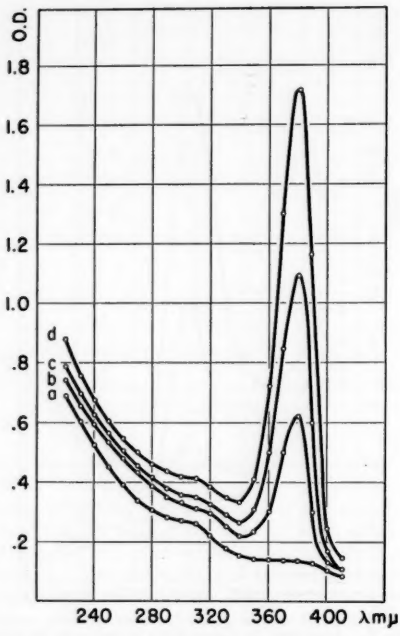

FIG. 5. ADDITION OF CHENODEOXYCHOLIC ACID TO NORMAL SERUM

Amounts added per ml. of serum: *a*) none, *b*) 0.015 mg., *c*) 0.040 mg., *d*) 0.080 mg.

ranged from 0 to 3.4 μg. per ml. for trihydroxy acids and 0 to 1.9 μg. per ml. for dihydroxy acids. These values are lower than those previously reported with this method (4, 6) because correction for background absorption has been made as described in the section on calculation. The validity of the occasional zero value is supported by the observation that occasionally extracts of as much as 50 ml. of serum from a healthy subject fail to contain any bile acids when analyzed by paper chromatographic techniques capable of detecting as little as 2 to 4 μg. of bile acid.

The serum bile acid concentrations in these healthy subjects did not vary significantly with age or sex and serial determinations showed no trends which could be correlated with eating or diurnal changes.

The trihydroxy-dihydroxy concentration ratio, to be discussed later, is significant only when the concentration of at least one bile acid type is elevated above the normal range. The average ratio for the 30 healthy subjects was 1.9 but the range extended from 0 to 6. This is because the error of the method and the standard deviations are of the same order of magnitude as the normal values, and consequently a wide range of ratios may occur in a healthy person by chance alone. The ratio becomes important, however, when the concentrations are elevated, as will be demonstrated in the following sections.

Patient material. In the two year period from July, 1955, through July, 1957, bile acid determinations were made on sera of 41 patients in whom the diagnosis of biliary obstruction or parenchymal liver disease was confirmed by laparotomy, biopsy, peritoneoscopy or autopsy and all of these are included in this report.

RESULTS

Biliary obstruction

There were 12 patients who had carcinoma or stone obstructing the bile ducts (Cases 1 to 12, Table II). All had elevated serum bile acid concentrations with a relatively greater increase in the trihydroxy acids resulting in a trihydroxy-dihydroxy ratio which was greater than 1 in all cases. An example of the ultraviolet absorption spectrum of the bile acids in the serum of a patient with biliary obstruction, Case 1, is shown in the center panel of Figure 6. For comparison, the spectrum of normal serum is shown in the panel at the left. It can be seen that the absorption maxima of trihydroxy acids (320 mμ) and dihydroxy acids (380 mμ) are increased above normal and that a greater rise has occurred in the trihydroxy fraction. The trihydroxy ratio in this patient was 7.8.

A similar pattern was also noted in sera of jaundiced patients with obstruction or damage to the smaller biliary radicals within the liver such as that which is sometimes produced by chlorpromazine (Case 13) or carbarsone (Case 14). During the obstructive phase of viral hepatitis (Cases 15 and 16), and especially in the "cholangiolitic" type of viral hepatitis (Case 17) as described by Watson and Hoffbauer (11), the serum bile acid pattern is the same as that of ordinary biliary obstruction.

Liver injury

Patients with severe liver cell injury, in contrast to those with biliary obstruction, had a striking and disproportionate increase in the concentration of dihydroxy bile acids, completely reversing the trihydroxy-dihydroxy ratio to less than one. An example of this is shown in the third panel of Figure 6. This patient (Case 18) had advanced alcoholic cirrhosis and lapsed into hepatic coma several weeks after her blood was drawn for the serum bile acid determinations. It should be noted that the total serum bilirubin con-

TABLE II

Serum bile acid concentrations and other laboratory studies in patients with hepato-biliary disorders

Case no.	Diagnosis	Serum bile acids: Trihydroxy	Serum bile acids: Dihydroxy	Ratio	Date	Serum bili. 1′/T*	Thymol turb.	Ceph. floc.	Serum cholesterol	Alk. p'tase KA units†	Serum protein alb./glob.	Comments
		μg./ml.				*mg. %*			*mg. %*		*Gm. %*	
1.	Carcinoma pancreas	82.0	10.5	7.8	9–22–55	19.8/31.9		0	392	67.5	2.6/5.2	Jaundice 6 wks.
2.	Carcinoma pancreas	74.4	6.7	11.1	12–14–55	5.0/8.5	2	0	155	57.4		Jaundice 3 days
3.	Carcinoma pancreas	24.9	6.0	4.2	2– 8–57	11.6/24.0	2	tr		52.6		Jaundice 7 wks.
4.	Carcinoma pancreas	24.9	2.1	12.0	4–30–56	2.5/6.3	11	0	176	62.6	2.8/3.6	Jaundice 22 wks.
5.	Stone, C.B.D.‡	4.5	2.6	1.7	11– 7–55	0.5/1.1	3	0		50.4		Jaundice 1 wk.
6.	Stone, C.B.D.	20.1	5.4	3.7	1–10–56	9.0/16.3	3					Jaundice 2 wks.
7.	Stone, C.B.D.	80.0	19.5	4.1	3–26–56	3.4/5.2	3	tr		37.0		Jaundice 1 wk.
8.	Stone, C.B.D.	3.5	1.0	3.5	2–23–56	0.3/0.6	3	1+		50.2		Jaundice 9 mo.
9.	Stone, C.B.D.	36.6	16.4	2.2	7–16–56	11.2/21.6	2	0			2.5/3.5	Jaundice 4 wks.
10.	Carcinoma metastatic, liver	45.0	9.1	4.9	12– 9–55	15.9/28.4	3	3+		86.3	2.7/3.3	Jaundice 3 wks.
11.	Carcinoma metastatic, liver	18.4	6.0	3.1	2– 8–56	4.3/7.0	4	0	167	67.5	2.9/3.6	Died 2–24–56
12.	Carcinoma C.B.D.	23.7	6.2	3.8	12–29–55	13.0/20.9	4	tr				Jaundice 3 wks.
13.	Cholangiolitis, chlorpromazine	33.6	12.6	2.7	8– 2–55	5.1/10.5	3	tr		54.4		Jaundice 1 wk.
14.	Cholangiolitis, carbarsone	76.1	31.5	2.4	12– 8–55	4.8/7.9	9	4+	276	55.6	3.0/3.8	Jaundice 4 days
		5.0	3.0	1.7	1– 6–56	1.3/2.5	8	2+	402	61.5	3.4/4.6	
15.	Hepatitis, viral	90.4	3.2	29.4	2–15–56	5.1/8.9	14	4+				Jaundice 1 wk.
		7.0	0.9	7.8	2–23–56	1.2/1.3	26	4+	288	28.8		
16.	Hepatitis, viral	22.1	11.3	2.0	2– 9–57	0.8/1.5	16	3+	153	23.4		Jaundice 2 days
17.	Hepatitis, viral	23.7	7.2	3.3	12–26–56	3.7/7.0	3	0		24.0	2.9/3.8	Jaundice 6 wks.
18.	Cirrhosis, portal	10.8	24.2	0.5	11–30–55	16.4/28.9	3	3+		19.4	2.6/4.1	
19.	Cirrhosis, portal	6.3	17.2	0.4	9–26–55	1.0/2.3	9	4+		15.8	1.4/4.6	Died 10–1–55
20.	Cirrhosis, portal	0	18.8	0	9–13–55	3.1/11.5		3+	106			Died 10–55
21.	Cirrhosis, portal	0	0.8	0	11–22–55	1.2/3.4	4	4+			1.6/5.3	Died 11–30–55
		0	10.5	0	11–30–55							
22.	Cirrhosis, portal	17.1	27.3	0.6	12– 2–55	18.6/32.8	4	tr	142	36.5	2.2/4.2	
		7.1	9.8	0.7	1–16–56	1.9/4.1	5	2+	191			
		0	21.9	0	12– 7–56	1.7/3.1	3	3+		27.8		Died 12–7–56
23.	Cirrhosis, portal	3.1	5.2	0.6	4–26–56	0.7/1.9	6	4+		12.7	1.8/5.2	Died 5–2–56
24.	Cirrhosis, portal	0.7	4.9	0.1	8–28–56	9.0/15.5	9	1+	93	30.0	1.9/3.8	Died 8–30–56
25.	Cirrhosis, portal	8.4	22.6	0.4	6–21–56	2.8/10.2		3+				
		2.2	11.3	0.2	1–19–57	0.7/1.7	8	4+		15.4	1.8/4.9	
		0.1	1.0	0.1	7– 9–57	1.0/3.1			148	6.5	2.0/4.0	Died 8–25–57
26.	Cirrhosis, portal	2.5	4.3	0.6	8–23–56	0.9/2.1	3	2+	99	10.8	1.8/3.0	Died 8–31–56
27.	Cirrhosis, portal	8.6	30.1	0.3	9–22–56	11.8/26.3	5	4+	44	29.3		
		5.9	22.0	0.3	9–25–56							Died 9–27–56
28.	Cirrhosis, portal	0	24.4	0	1–25–57	3.6/8.9	5	4+	117	11.5	1.7/5.1	Died 1–27–57
29.	Cirrhosis, portal	0	2.4	0	11–27–56	0.3/1.0	5	3+			2.6/4.0	Died 4–17–57
30.	Cirrhosis, post necrotic	18.2	45.5	0.4	8– 2–56	9.1/20.0	6	3+	115			Died 1–57
31.	Cirrhosis, post necrotic	6.8	9.1	0.8	5– 1–56	0.5/1.7	17	4+	182	14.8	2.6/4.5	Died 10–24–57
32.	Cirrhosis, post necrotic	0	55.0	0	8–16–56	9.4/13.8	3	2+	39	23.6	3.2/2.5	
		0	45.4	0	8–28–56	16.3/28.8	3	3+		23.3		Died 9–10–56
33.	Cirrhosis, post necrotic	5.5	43.5	0.1	9–25–56	7.7/14.7	19	4+	114	18.4	1.8/5.4	Died 10–2–56
34.	Cirrhosis, post necrotic	0	13.1	0	12–20–56	1.5/3.5	7	4+	85		2.2/3.4	Died 2–12–57
35.	Cirrhosis, biliary	29.8	4.6	6.5	11–22–55	8.7/14.8	12	4+		82.8		
		0	2.5	0	12–27–56	9.2/19.1	9	3+			2.0/3.0	Died 12–31–56
36.	Cirrhosis, biliary	0.2	0.5	0.4	1– 2–57	1.9/4.7	4	3+		35.8	3.4/3.1	Died 3–30–57
37.	Cirrhosis, biliary	2.8	3.2	0.9	4–30–56	9.3/18.0	2	2+		40.5	2.4/3.2	Died 4–28–56
38.	Hepatitis, viral	25.8	30.4	0.8	4–27–56	8.8/16.5	12	4+	116	32.3	2.7/3.3	Died 5–16–56
		0	11.0	0	5– 6–56	15.9/28.4	9	4+			2.1/3.3	
39.	Hepatitis, viral	23.9	31.7	0.8	7–18–56	3.5/5.1	10	4+		24.8	2.1/3.3	
		5.9	14.4	0.4	7–23–56	5.6/10.1			78			Died 8–10–56
40.	Cirrhosis, portal	3.1	2.0	1.6	11–22–55	0.8/2.5		1+				
		4.7	18.6	0.3	1–15–56	0.8/2.2	4	4+			2.3/4.8	
		4.4	2.0	2.2	1–25–56	0.4/0.5	3	0				
41.	Cirrhosis, portal	1.4	3.9	0.4	6–21–57	2.8/4.5	5	3+				
		73.3	14.7	5.0	6–27–57	2.0/3.4	5	3+		40.5		

* One minute/total.

† Alkaline phosphatase, King-Armstrong units.

‡ Common bile duct.

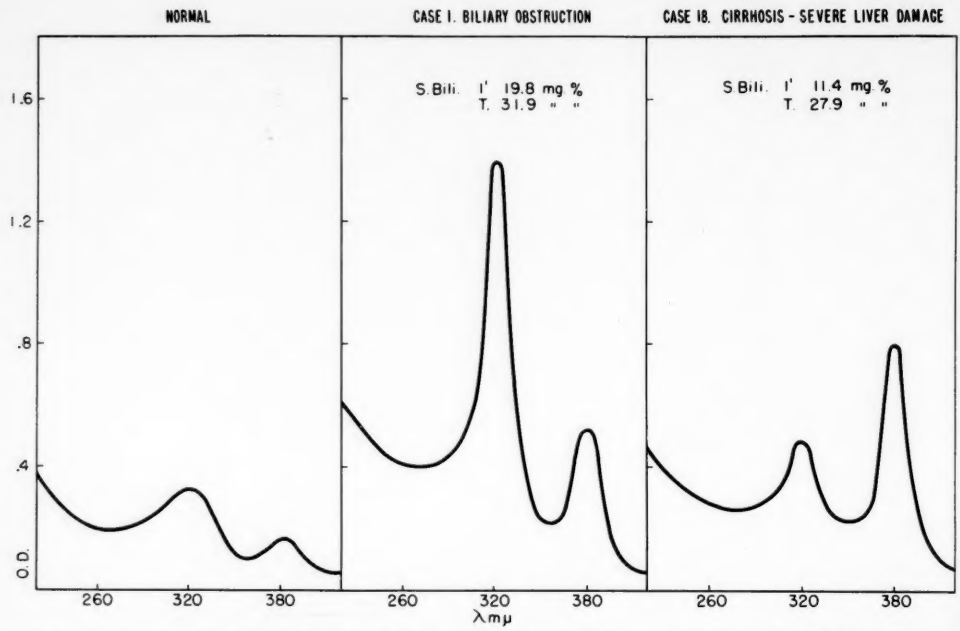

FIG. 6. ULTRAVIOLET ABSORPTION SPECTRA OF BILE ACIDS IN SERA OF PATIENTS WITH (LEFT) NORMAL SERUM, (CENTER) BILIARY OBSTRUCTION AND (RIGHT) LIVER DAMAGE

centrations of the two patients shown in Figure 6 are of the same order of magnitude.

The severity of the liver damage associated with this reversed bile acid ratio has been clearly demonstrated to us by the fact that slightly less than one-half the patients died within a few weeks or months following this reversal (Cases 19 to 39). An additional one-fourth are in the terminal stages of their illness.

The reversed ratio also occurs during acute liver injury and will return to one or greater than one as the patient recovers. An example of this is Case 40. This man had cirrhosis associated with chronic alcoholism. He was admitted to the hospital in November, 1955, because of a peptic ulcer. The bile acids were very slightly elevated, but the ratio was 1.6. He returned the following January after a drinking bout, in a state of acute alcoholism and impending delirium tremens. The injury to his liver was reflected by a rise in the dihydroxy bile acids causing a reversal of the trihydroxy-dihydroxy ratio to 0.3. The Hanger test became 4 + but the serum bilirubin changed very little. With medical care and gradual recovery, the ratio returned to a value of 1.1 10 days later, and the Hanger test became negative. It should be emphasized here that if only cholic acid had been measured, relatively little change would have been noted.

The prognostic value of the trihydroxy-dihydroxy bile acid ratio is illustrated quite well by Cases 25 and 33. Case 25 was a man with cirrhosis associated with alcoholism, who had ascites and was facing hepatic coma upon his admission in December, 1955. Following a period of hospital care, salt restriction, diuresis, bed rest, a good diet and abstinence from alcohol, the patient became more alert and the ascites disappeared. This improvement was reflected by a fall in the serum bilirubin and bile acid concentrations. The ratio, however, remained reversed (0.7) and the Hanger test changed from trace to 2 +. The patient returned one year later in deep coma and died a few hours after admission; at this time, the trihydroxy-dihydroxy ratio was zero because of the complete absence of cholic acid.

The second example, Case 33, was a 44 year old woman admitted in late September, 1956, having

had jaundice for two months. No stigmata of chronic liver disease were present and there was no ascites. The patient became confused on the fourth hospital day and died in coma on the eighth hospital day. The serum bile acids were determined two days after admission while the patient was alert, active and had no signs to suggest that she would die in a few days. The approximately 50-fold increase of dihydroxy acids above normal, however, with a comparatively minor rise of trihydroxy acids, strongly suggested the extensive liver damage which was subsequently found at autopsy as shown by the photomicrograph in Figure 7. A similar disproportionate rise in the serum dihydroxy acids was noted in Case 32, a 19 year old girl. In this instance, the ultraviolet absorption spectrum of the serum resembled very closely that of pure chenodeoxycholic acid, which was easily separated from her serum by means of paper chromatography. This girl died in hepatic coma four weeks later.

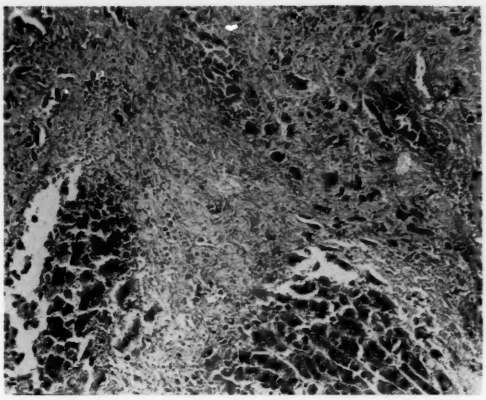

FIG. 7. PHOTOMICROGRAPH OF LIVER FROM CASE 33 SHOWING EXTENSIVE LIVER DAMAGE

DISCUSSION

From the material presented, there are several observations which deserve emphasis. The first of these is that the differential analysis of the two bile acid types provides more valuable information than the measurement of only a single bile acid. This is so because the ratio of the concentration of the two major types of bile acids is of greater importance than their individual concentrations. If only the trihydroxy bile acid concentration (mostly cholic acid) is compared with the other liver function studies shown in Table II, it is clear that relatively little helpful information is obtained concerning either the liver's condition or the cause of the jaundice. This was also the conclusion Sherlock and Walshe made from their careful study of serum cholates in liver disease (1). When both types of bile acids are measured, however, differences in the quantitative relationships between the two types, simply expressed as the trihydroxy-dihydroxy ratio, immediately become of some importance, especially as a measure of the extent of liver injury. When liver damage is extensive, the ratio ranges from less than 1 to 0. With biliary obstruction, on the other hand, serum bile acid concentrations increase, but the ratio will be greater than one, sometimes reaching a value of 20 to 30 or more. When the obstruction is prolonged to the point of extensive liver damage, however, as it was in Case 35, serum bile acid concentrations fall, and the ratio is lowered.

It should be stressed at this point that serum bile acid determinations as described in this paper will not disclose the location of the biliary obstruction and thus will not provide an answer to the often difficult question of whether the jaundice must be dealt with surgically or treated medically; in other words, whether the disease involves the smaller biliary radicals within the liver, or the extrahepatic bile ducts.

Pruritis

The relationship of bile acids to the pruritis of jaundice is by no means clarified by this study. Bile acid concentrations were found to be elevated in the sera of all jaundiced patients who had pruritis, but no pruritis occurred at any time in several patients who had very high serum bile acid concentrations (Cases 2, 33).

In order to study this point more fully, a patient with alcoholic cirrhosis (Case 41) was fed one Gm. of pure cholic acid in a gelatin capsule daily for seven days, at which time the serum trihydroxy acid reached a concentration of 73.3 μg. per ml., a value exceeding that which accompanied itching in other individuals, but no pruritis occurred in this patient. It is well known, of course, that the administration of commercial bile salt preparations to some jaundiced subjects will pro-

duce pruritis (12), but practically all such preparations, except those composed of dehydrocholic acid only, contain other constituents of the bile besides bile acids. Obviously the question of whether elevated serum bile acid concentrations are only associated with pruritis or actually cause it will require additional information before a definite answer can be provided.

The bile acid ratio

Mechanisms responsible for the reversed bile acid ratio in severe liver injury have not been elucidated. From the information at hand it is not unreasonable to suspect that the activity of a 12 α-hydroxylating enzyme is deficient. Bergström and Sjövall (13) and Mahowald and associates (14) have shown in rats that chenodeoxycholic acid is not an intermediate in the formation of cholic acid. Bergström (15) has also demonstrated that 3α, 7α-dihydroxycoprostane is readily converted to cholic acid as well as chenodeoxycholic acid in rats. If these pathways of bile acid synthesis in the rat are also important routes in the human liver then the increased serum concentration of chenodeoxycholic with liver injury may result from degradation of the side chain of the molecule to a 24 carboxy acid before hydroxylation at the 12α position can occur. Evidence to support such speculation is provided by the observation (16) that the 12 position is less vulnerable to oxidation than the 7 position, partly because of hindrance by the angular methyl group at Carbon 13 (17). The enterohepatic return of predominantly dihydroxy bile acids to the damaged liver, which cannot re-excrete them normally, would also contribute to the quantity of dihydroxy acid in the serum. A more prolonged exposure of bile acids to these hydroxylating enzymes has been suggested by Rudman and Kendall (5) to explain the increased proportion of trihydroxy acids in serum with biliary obstruction.

SUMMARY AND CONCLUSIONS

1. A quantitative method for the simultaneous differential determination of serum trihydroxy and dihydroxy bile acids is described. Normal values have been established for a group of healthy subjects and variations from the normal have been studied in 41 patients with various hepatobiliary disorders.

2. With severe liver injury a disproportionate increase in the dihydroxy bile acid concentration reduced the trihydroxy-dihydroxy ratio to less than 1.0.

3. Persistence of this reversed ratio is frequently followed by coma, death or both. When the ratio is only temporarily reversed, as with acute or transient liver injury, improvement or recovery of the patient is paralleled by a rise in the ratio towards a value of 1.0 or more. This is helpful in estimating the extent of liver injury and monitoring its course.

4. With biliary obstruction, whether intra- or extrahepatic, the serum bile acid concentrations are increased but the ratio remains greater than 1.0.

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THE METABOLISM OF ALBUMIN–BOUND C^{14}–LABELED UNESTERIFIED FATTY ACIDS IN NORMAL HUMAN SUBJECTS

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Recent studies have indicated that the unesterified fatty acids in plasma (UFA) may represent a major form in which fat is transported to tissues for utilization as a source of energy. The evidence indicating the metabolic importance of UFA includes a close correlation between plasma UFA concentration and nutritional state (1, 2), direct evidence of removal of UFA by the heart and liver in fasting humans (2, 3), and the early participation of UFA in chylomicron metabolism (4). These studies have indicated that plasma UFA turnover must occur at a rapid rate. This fact has been established in recent studies on dogs (4–6), humans (7, 8), rabbits (9), and rats (10). The purpose of this paper is to describe the measurement of the rates of plasma turnover and oxidation of albumin-bound C^{14}-labeled unesterified fatty acids in normal human subjects. A tentative scheme for UFA metabolism in man is also presented.

METHODS

The C^{14}-carboxyl labeled acids used in these studies included palmitic and linoleic acids obtained from the Nuclear Instrument and Chemical Corporation, Chicago, Ill., and oleic acid from Tracerlab, Inc., Boston, Mass. The palmitic and linoleic acids were subjected to 25 and 50 tube counter-current distribution, respectively, using the solvent systems of Ahrens and Craig (11). With both acids, 95 to 98 per cent of the radioactivity was distributed in a single symmetrical peak. In the case of the linoleic acid, the distribution of radioactivity corresponded exactly to that of added unlabeled linoleic acid, obtained from the Hormel Institute, and determined by titration. The remaining radioactivity was present in material with partition coefficients corresponding to very polar short chain acids. The method of Howton, Davis and Nevenzel (12) used for preparation of the linoleic acid has been found to yield as much as 15 per cent trans isomers. The amount of labeled linoleic acid available was too small to permit spectroscopic analysis for such isomers. The oleic acid was analyzed by vapor phase chromatography, through the courtesy of Dr. E. H. Ahrens. The radioactivity was distributed as follows: 89.5 per cent as oleic acid, 6.5 per cent as stearic, palmitic or longer chain acids, 2.1 per cent as palmitoleic, and the remaining 1.8 per cent as 12 to 15 carbon acids. The approximate specific activities of the three acids were: palmitic, 2.5 mc. per mM; linoleic, 4.46 mc. per mM; and oleic, 0.43 mc. per mM.

The labeled acids were prepared for injection in the following manner. The acids were dissolved in aqueous ethanol containing a molar equivalent amount of K_2CO_3 and heated briefly at 100° C. to evaporate the ethanol. While still warm, the aqueous solutions were added to approximately five volumes of a 25 per cent solution of human serum albumin, obtained from the American Red Cross. The final solutions, containing less than 1 mole added fatty acids per mole albumin, were optically clear. Individual doses were placed in sterile vials, cultured and kept frozen until use.

Subjects. Twenty-one male and two female patients at the Clinical Center, ranging in age from 18 to 76 years, were utilized as "normals" for these studies. Eighteen of these patients were "normal control volunteers," admitted for research studies only, who had no evidence of organic disease. The five remaining patients had been admitted for study of unrelated disorders, and had no clinical evidence of abnormal lipid metabolism. With the exception of one patient, whose serum triglycerides were 300 mg. per cent, all the subjects had normal levels of serum total and esterified cholesterol, phospholipids and triglycerides. Turnover studies were also made in several patients with idiopathic hyperlipemia, essential hypercholesterolemia with xanthoma tendinosum, diabetic acidosis, or the nephrotic syndrome. The data obtained in the latter patients were not utilized, however, in the formulation and interpretation of the normal metabolism of UFA in the human and are referred to only when specifically stated.

The nutritional status of the subjects was varied to achieve a wide range of plasma UFA concentration at the time of the test. In general, fasting or carbohydrate-loading was employed. The fasting subjects were usually permitted no food after the evening meal, roughly 16 hours before the injection of labeled substrate. Some of the subjects, including all upon whom measurements of radioactivity in blood and expired air were extended beyond three hours, were fasted a total of 20 to 24 hours before the test, and kept without food until the completion of observations up to 24 hours later. The "carbohydrate-loaded" subjects were fed approximately 250 grams

of carbohydrate as Karo® syrup diluted with water, in divided doses beginning two hours before and continuing during the period of observations. In several instances glucose was also administered intravenously during the test period. During the first hour of observations, the subjects rested on a table. An inlying needle was placed in a brachial artery to facilitate rapid collection of blood samples. A mouthpiece connected to a low-resistance, two-way respiratory valve permitted inspiration of room air and expiration into a train of Douglas bags.

After blood was removed for determination of plasma lipids and zero time plasma UFA concentration, the labeled UFA-C^{14}-albumin solution, varying from 3 to 15 ml. in volume, was rapidly injected intravenously. The dose administered varied from 0.001 to 0.005 mc. and represented 0.005 to 0.01 mEq. added UFA. Blood samples were then taken rapidly at 2, 3, 4, 5, 6, 8 and 10 minutes and varying intervals thereafter for 60 minutes. The arterial needle was then removed and subsequent blood samples taken at longer intervals from a peripheral vein. An additional blood sample was usually removed at 10 minutes for a second determination of UFA concentration. About 10 seconds were required to remove each of the 11 ml. blood samples.

Simultaneously with the injection of the labeled substrate, the collection of expired air was begun. Routinely, air was collected for 2 minute periods for 10 minutes, then for 5 minute periods for a further 50 minutes. Subsequent 5 to 10 minute samples were collected at intervals up to 24 hours. When the observations were extended for 24 hours, the subjects remained at rest during the day and were awakened several times during the night for collection of air and blood samples. Blood samples were collected in dry syringes, transferred to tubes containing heparin, mixed thoroughly and immediately placed at 0 to 4° C. until analytical procedures could be begun.

For measurement of UFA radioactivity and concentration, the chilled blood samples were centrifuged and the plasma lyophilized and extracted according to the method previously reported (3). It was determined initially that such extracts of plasma obtained 15 minutes and longer after administration of the tracer acids contained significant radioactivity in neutral lipids. The iso-octane extracts of plasmas taken after 10 minutes were therefore extracted with 0.1 N NaOH in 50 per cent ethanol according to the procedure of Borgström (13). The aqueous extracts were then acidified, and the fatty acids re-extracted with iso-octane. Separate aliquots of the final iso-octane phase were used for titration and for counting. UFA specific activity was expressed as counts per minute per mEq. titratable acidity. Radioactivity was also determined in the UFA-free extracts. In the early (2 to 10 minute) samples, the entire iso-octane extract was assayed for radioactivity. UFA specific activity was then calculated, using the mean of the separately determined zero time and 10 minute UFA concentrations.

An aliquot of the labeled fatty acid-albumin solution was extracted in acetone-ethanol 1 : 1, and aliquots of this were taken for measurement of the total radioactivity administered.

Carbon dioxide content, radioactivity and specific activity in the expired air samples were determined by a method previously described (14).

In the preparation of the samples for assay of radioactivity, the solvent extracts were evaporated at steam bath temperature. Twenty-five mg. of unlabeled palmitic acid was added to the vials containing UFA before evaporation to minimize loss of radioactivity through sublimation. Two ml. aliquots of the amine-carbonate solutions for $C^{14}O_2$ assay were placed directly in the counting vials. Fifteen ml. of phosphor solution (400 mg. per cent diphenyloxazole in toluene) was added to each vial. Assays were made at $-10°$ C., using a Packard Tri-Carb Scintillation spectrometer. To permit direct comparison with the assays of lipid radioactivity, the radioactivity in the $C^{14}O_2$ samples was corrected for quenching through use of an internal standard. The anticipated standard error in assay of radioactivity of plasma UFA rose from less than ± 1 per cent in the samples taken during the first 10 minutes to ± 10 to 25 per cent in the samples taken at seven hours. The maximum anticipated standard error for the $C^{14}O_2$ assays was ± 10 per cent.

All data were subsequently corrected by multiplying by the factor: $\frac{\text{body weight (in Kg.)} \times 10^5}{\text{radioactivity administered (in cpm)}}$. The results so expressed therefore correspond in each subject to those which would have been obtained at a constant dose of 10^5 cpm per Kg. body weight.

RESULTS

Disappearance of UFA-C^{14} from the plasma

The early disappearance of radioactivity from the plasma UFA after injection of palmitic acid-1-C^{14} is shown in Figure 1. In this subject blood samples were obtained by collecting sequential five second samples from the arterial needle for the first three minutes after injection. The concentration of radioactivity declined exponentially from the second to the eighth minute. Intravascular mixing appeared to be complete within the first two minutes. This was consistent with the known behavior of the albumin-bound dye, T-1824, immediately following its intravenous administration (15). In most subjects, a semilogarithmic plot of the concentrations of radioactivity against time began to deviate from linearity after the fourth or fifth minute. The disappearance curve of UFA radioactivity thereafter rapidly became more complex (Figure 3). As will be discussed later, much of this complexity appeared to repre-

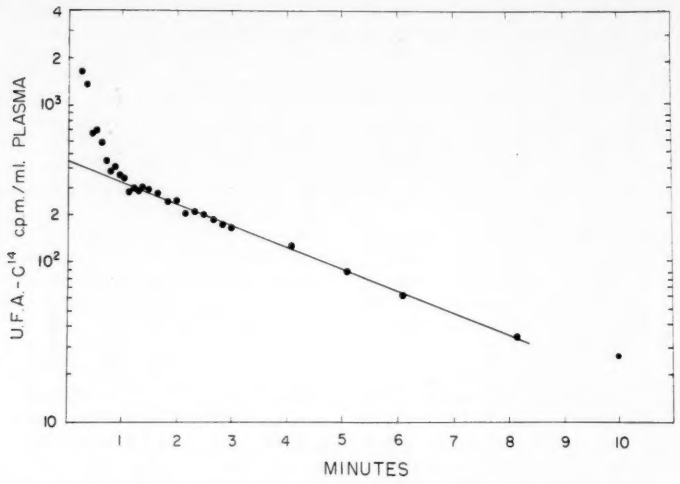

FIG. 1. THE DISAPPEARANCE OF PALMITATE-1-C^{14} FROM THE PLASMA IN A NORMAL SUBJECT FASTED FOR 42 HOURS

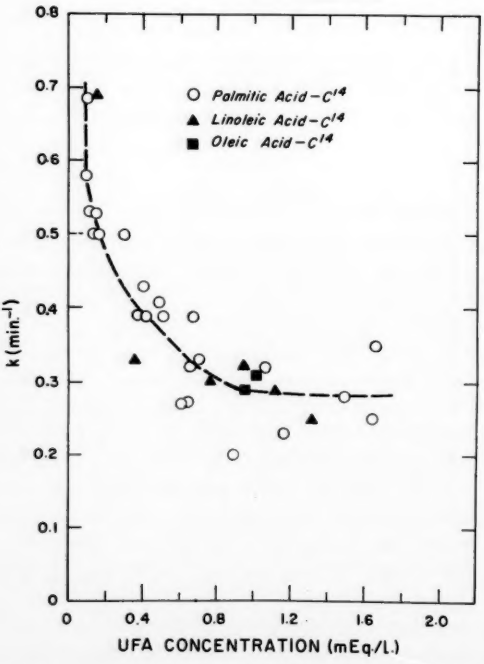

FIG. 2. COMPARISON OF THE FRACTIONAL TURNOVER RATE OF PLASMA UNESTERIFIED FATTY ACIDS (UFA) WITH PLASMA UFA CONCENTRATION

sent reflux of radioactive UFA back to the blood. The initial slope of this curve was felt to represent the most accurate measurement of the rate of disappearance of radioactive UFA from the plasma. Therefore, the straight line fitted to the semilogarithmic plot of the UFA specific radioactivity obtained at the second, third and fourth minutes was used to calculate the fractional turnover rate of plasma UFA. In 30 such determinations made in subjects whose plasma UFA concentration varied 19-fold (0.09 to 1.71 mEq. per L.), the half periods of decay ranged from 1.0 to 3.4 minutes. The relationship of the fractional turnover rate of plasma UFA to the plasma UFA concentration is shown in Figure 2. It was of interest that the rate constants calculated for the disappearance of palmitic and linoleic acids at various concentrations, and for oleic acid at the single concentration tested, were very similar. From Figure 2, it will be observed that the fraction of the plasma UFA removed per minute is approximately one-quarter of the total plasma pool at UFA concentrations greater than 1 mEq. per L. In one diabetic in moderate acidosis, not included in the data presented, the same fractional turnover rate was observed at a plasma UFA concentration of 2.09 mEq. per L. As plasma concentrations fell

below 1 mEq. per L. the fractional turnover rate appeared to be concentration dependent and increased sharply with decreasing UFA concentration. The quantity of UFA removed from the plasma per minute (flux of UFA from plasma, in mEq. per minute) was calculated by dividing the product of plasma UFA concentration and the estimated plasma volume of the subjects (taken as 3.5 per cent of body weight) by the fractional turnover rate. Fluxes so calculated varied from 0.14 to 1.50 mEq. per minute. These calculations included the assumption that the fractional turnover rates of all other unesterified fatty acids were similar to those of palmitic, oleic and linoleic acid.

The zero time mixing volume for the injected radioactive UFA was estimated from the intercept of the extrapolated two to four minute total radioactivity-time curve and the dose administered. These mixing volumes averaged 30 per cent greater than the estimated plasma volume. The discrepancy between zero time mixing volume and plasma volume was found to be of comparable magnitude in three subjects in whom plasma volume was experimentally determined by T-1824 dye dilution. The finding of a "mixing volume" greater than plasma volume was consistent with the rapid removal of UFA, since considerable loss of radioactivity would be expected to occur before mixing was complete.

The time course of plasma UFA specific activity for 22 hours after injection of labeled acids is shown in Figures 3 and 4. Figure 3 represents composite data from four subjects and Figure 4 from three subjects. The specific activity-time curves for UFA and $C^{14}O_2$ after injection of oleate-C^{14} as determined in two subjects were not significantly different from those obtained after palmitate-C^{14} (Figure 3). With these two acids,

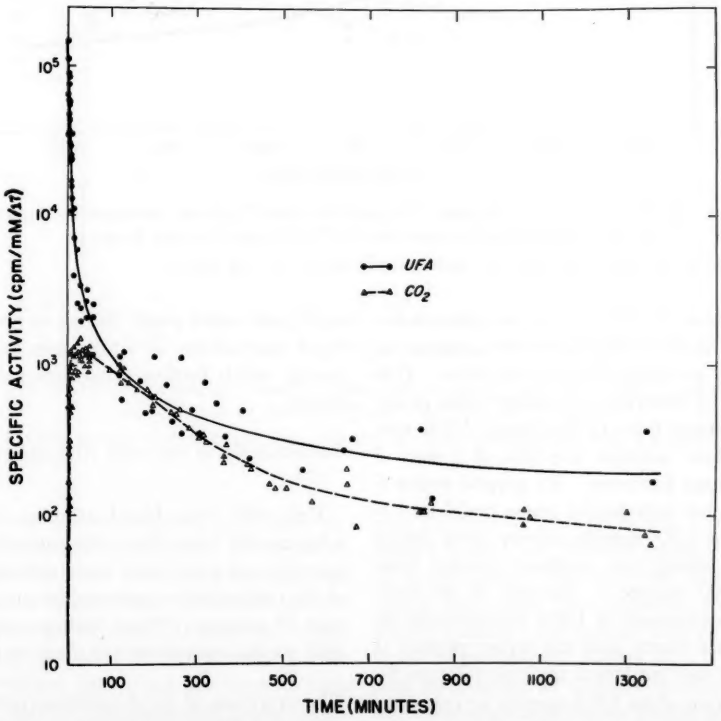

FIG. 3. SPECIFIC ACTIVITY IN PLASMA UFA AND EXPIRED CO_2 AFTER INTRAVENOUS INJECTION OF ALBUMIN-BOUND PALMITIC ACID-1-C^{14} IN FOUR FASTING SUBJECTS
The UFA specific activities only have been divided by 17 (see text).

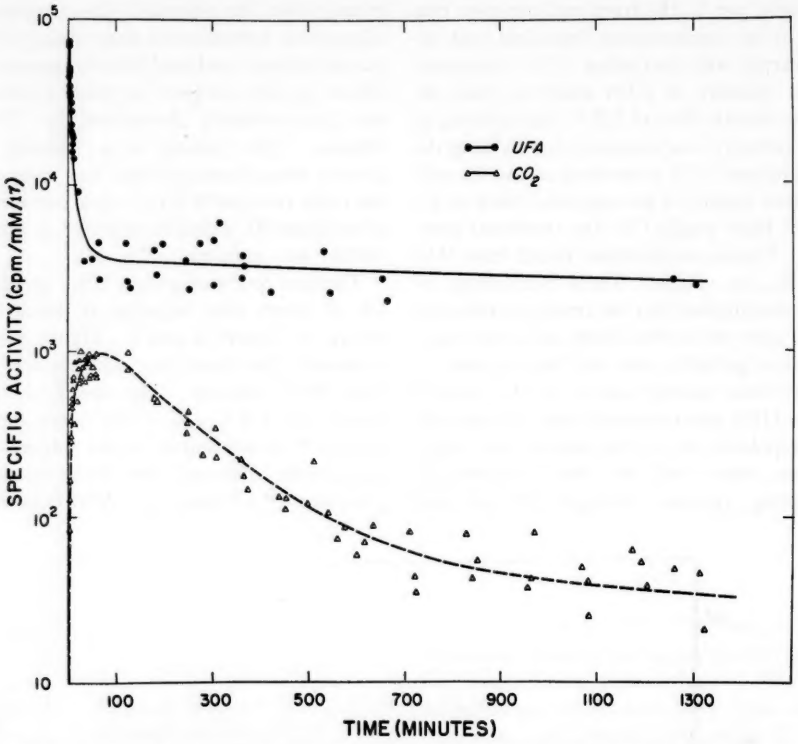

FIG. 4. SPECIFIC ACTIVITY IN PLASMA UFA AND EXPIRED CO_2 AFTER INTRAVENOUS INJECTION OF ALBUMIN-BOUND LINOLEIC ACID-1-C^{14} IN THREE FASTING SUBJECTS

The UFA specific activities only have been divided by 17 (see text).

the concentration of UFA-C^{14} in the plasma declined rapidly in 30 to 60 minutes to a concentration of about 1 per cent of that at zero time. This was followed by progressively slower rates of decline. The general form of the plasma UFA specific activity-time function was that of a sum of exponential decay processes. By graphic subtraction, at least five exponential terms could be obtained from the UFA specific activity curve shown in Figure 3, having rate constants varying from 0.27 to 0.00032 minute^{-1}. Because of the inaccuracy in measurement of UFA radioactivity after five to seven hours, only the earlier portion of the curve was used for kinetic analysis (Figure 5). The later portion of the UFA specific activity-time curve derived from linoleic acid-C^{14} (Figure 4) had a different form from that obtained with either palmitic or oleic acid. After injection of linoleic acid there was a rapid decline to about a level of 1 per cent of the zero time specific activity, following which further decay proceeded extremely slowly.

Radioactivity in red cells after injection of UFA-C^{14}

Red cells from blood samples removed from subjects one hour after administration of labeled palmitic and oleic acids contained only 1 per cent of the radioactivity contained in an equivalent volume of plasma. These findings were consistent with similar measurements made in the dog (16).

The excretion of UFA carboxyl carbon-14 in the expired CO_2

The oxidation of labeled fatty acids introduced into the plasma proceeded rapidly. Specific ac-

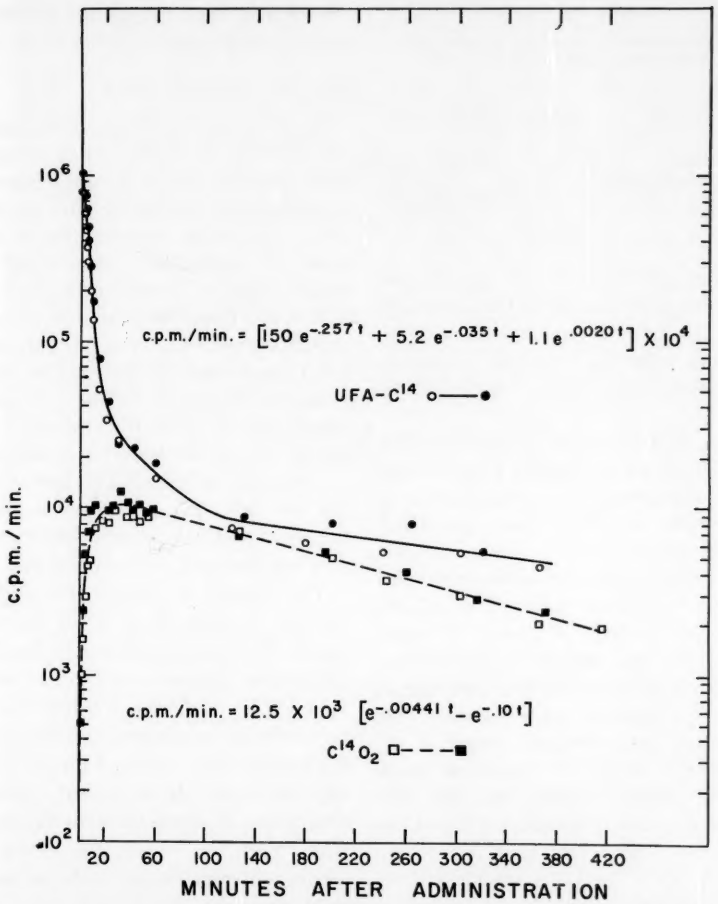

FIG. 5. FLUX OF LABELED CARBOXYL CARBON OF PALMITIC ACID-1-C^{14} FROM PLASMA AS UFA AND IN EXPIRED CO_2 IN TWO FASTING SUBJECTS

Open symbols represent data from one subject; solid symbols, data from the second subject.

tivity-time curves for the expired CO_2 after injection of palmitic acid also appear in Figure 3. The composite data from two subjects given oleic acid were practically identical. Maximum specific activity was reached in about 30 minutes. After injection of linoleic acid, the maximum specific activity was reached after about one hour and the peak specific activity was somewhat lower (Figure 4). The specific activity-time function for all three acids could be expressed in the general form: $cpm/mM = Ae^{-at} + Be^{-bt} + Ce^{-ct}$. The rate constant b, predominating from the first to eighth hours, was 0.0069 $minute^{-1}$ for palmitic acid (calculated from the curve fitted to the composite data from four patients, Figure 3) and 0.0065 $minute^{-1}$ for linoleic acid (composite data from three patients, Figure 4). The total fraction of radioactive carboxyl carbon excreted by one hour after injection is shown in Table I. In the seven patients in whom extended measurements were made, the fraction excreted by 22 hours ranged from 31 to 64 per cent of the injected

TABLE I

The appearance of carboxyl carbon-14 in expired air after injection of labeled unesterified fatty acids (UFA)

Acid	Nutritional status	No. of subjects	Per cent dose C^{14} excreted in 1 hr.
Palmitic	Fasting	11	9.4 (5.7–13.3)
Palmitic	Carbohydrate-fed	3	4.7 (2.7–6.0)
Oleic	Fasting	2	10.0 (8.2–11.8)
Linoleic	Fasting	5	6.3 (5.3–8.2)

dose. In the one patient given both palmitic and linoleic acids with measurement of CO_2 excretion for as long as 22 hours, the total amount of radioactivity excreted following injection of linoleic acid was two-thirds of that obtained from palmitic acid.

For purposes of comparison, the plasma UFA specific activities shown in Figures 3 and 4 were divided by 17, representing an approximation of the average number of carbon atoms per UFA molecule in the plasma (17). The assumption that a labeled carboxyl carbon in the injected acids will undergo an "obligatory dilution" of 17-fold is based on the observations in rats of Weinman and associates (18) that carbons 1, 6 and 11 of injected palmitic acid emerge at the same rate in the expired CO_2. Such an approximation does not allow for any undetermined amount of recycling of fragments of the UFA molecule which may occur. For palmitic and oleic acids the "corrected" specific activities of plasma UFA and expired $C^{14}O_2$ were similar for about two to six hours, following which the UFA specific activity again became greater. Following injection of linoleic acid the UFA specific activity was always many times greater than that of the expired CO_2.

Recycling of plasma UFA

Inspection of the plasma UFA specific activity curves shown in Figures 3 and 4 suggests that labeled carboxyl carbon from the injected C^{14} acids is returning to the plasma after its disappearance. More meaningful computations to ascertain this point are obtainable by converting the observed specific activity measurements into the form of flux of C^{14} from the plasma as UFA or into the expired air as $C^{14}O_2$. For this purpose, the plasma UFA specific activity (in cpm per mEq.) at time t may be multiplied by the experimentally estimated flux of UFA from plasma (in mEq. per minute) as defined above, to obtain the flux of C^{14} (in cpm per minute). The mean output of CO_2 (in mM per minute) is likewise used to convert the observed $C^{14}O_2$ specific activities (in cpm per mM) to flux of C^{14} in expired air.

The changes in isotopic flux with time calculated for two subjects receiving palmitic acid-1-C^{14} are plotted semilogarithmically in Figure 5. A single curve has been fitted to the combined values for UFA-C^{14} and for $C^{14}O_2$ from each patient and an analytical expression, consisting of a sum of exponential terms, derived for the curves. These expressions may be integrated over the period of observation to obtain the total flux during this period. Such total fluxes for several subjects receiving different labeled acids are summarized in Table II.

TABLE II

Isotope data in six fasting patients

Patient	Status	C^{14}-acid	Plasma UFA at outset	Plasma UFA flux	CO_2 output	Amount C^{14} administered	Isotopic flux* in Plasma UFA	Isotopic flux* in CO_2
			mEq./L.	*mEq./min.*	*mM/min.*	*10^6 cpm*	*10^6 cpm*	
M. B.	Fasting	Palmitic	0.61	0.42	8.2	7.2	10.5†	2.3†
L. J.	Fasting	Palmitic	1.16	0.74	9.2	7.8		
P. R.	Fasting	Oleic	0.95	0.63	10.2	7.0	10.1	2.7
B. S.	Fasting	Linoleic	0.95	0.67	13.0	7.6		
G. G.	Fasting	Linoleic	1.32	0.87	15.2	7.5	26.4†	2.6†
Q. W.	Fasting	Linoleic	1.12	0.81	8.3	7.2		

* Flux of $C^{14} = \int_0^t \gamma\, dt$; where t = 420 minutes, γ = the sum of exponential terms fitted to the curves for the isotopic flux in plasma UFA ($\gamma = \gamma_a$), or in expired $C^{14}O_2$ ($\gamma = \gamma_b$), observed for the first 420 minutes following administration of the tracer acid.

† Data from these subjects were combined and fitted with a single curve from which γ_a and γ_b, respectively, were derived.

From inspection of these data, it is obvious that within seven hours after injection of labeled palmitic and oleic acids to fasted subjects, about 50 per cent more radioactivity than was administered has passed through the plasma. Since the plasma UFA still contains slowly declining radioactivity at 22 hours (Figure 3), it is certain that, were an analytical expression (γ_a, Table II) for isotopic flux in plasma UFA available which contained sufficient exponential terms adequately to describe this late decay, the integrated flux, $\int_0^\infty \gamma_a \, dt$, would be several times the injected dose. This assumes that the fractional turnover rate of plasma UFA remains the same and that the radioactivity in the plasma UFA is contained in the acid in which it was injected. It is reasonable to assume that some recycling of labeled carboxyl carbon may occur in acids other than the original labeled substrate, and this factor is being evaluated by chromatographic techniques.

As was previously mentioned, linoleic acid-1-C^{14} behaves differently from palmitic and oleic acid, undergoing far more recycling into the plasma compartment (Table II). It will be noted that two of the three subjects given this acid had unusually high total CO_2 outputs. Observations on a slightly larger group (Table I) indicate that linoleic acid carboxyl carbon is excreted more slowly than that from palmitic and oleic acids.

Effect of nutritional state on plasma turnover and oxidation of UFA

Since there is considerable evidence indicating the precedence of glucose oxidation over that of fat both in humans and other animals (19), it was considered pertinent to compare UFA metabolism in fasting and carbohydrate-loaded subjects. The flux of plasma UFA in fasted and carbohydrate-loaded subjects is shown in Table III.

Although the mean plasma UFA concentration in the carbohydrate-fed subjects was one-sixth of that in the fasted subjects, the greater fractional turnover rate of plasma UFA at the lower plasma UFA concentrations tended to equalize the two groups with respect to UFA flux. The total flux of plasma UFA in carbohydrate-fed subjects averaged about one-third of that in individuals fasted an average of 16 hours. The oxidation of UFA, once introduced into the plasma, proceeds more slowly after carbohydrate loading, but is by no means eliminated (Table I). It has been shown in rats (20, 10) that carbohydrate feeding virtually abolishes oxidation of intravenously injected palmitic acid. It is of interest that in man it was not found possible to prevent appreciable oxidation of plasma UFA by supplying an excess of calories as carbohydrate. One subject received a high-calorie diet containing over 300 Gm. of carbohydrate daily for three days, plus an intravenous infusion of 100 Gm. of glucose with insulin during the first two hours after injection of palmitic acid-1-C^{14}. His total output of radioactivity within seven hours was about one-third of that expired by fasted subjects receiving palmitic acid.

TABLE III

Effect of nutritional state on flux of palmitic acid-1-C^{14} from the plasma

Status	No. of subjects	Plasma UFA		
		Concentration	$T_{\frac{1}{2}}$	Flux
		mEq./L.	*min.*	*mEq./min.*
Fasting	18	0.79 (0.30–1.65)	2.2 (1.4–3.4)	0.66 (0.32–1.50)
Carbohydrate-fed	6	0.13 (0.09–0.16)	1.3 (1.0–1.4)	0.23 (0.14–0.49)

Comparison of the rate of CO_2 excretion (in mM CO_2 per minute ÷ 17) with the total flux of UFA from the plasma was also made (Figure 6) for fasting and carbohydrate-loaded subjects. In fasted subjects, the flux of UFA from the plasma was approximately equivalent to the rate of CO_2 excretion. As will be discussed presently, this does not imply that the only source of carbon dioxide during fasting is the oxidation of plasma UFA. In the carbohydrate-loaded subjects, the rate of CO_2 excretion was severalfold greater than the flux of UFA from the plasma.

Radioactivity in the neutral lipid fraction

The combined extraction procedure used also permits assay of radioactivity in a UFA-free extract. The neutral lipids include all of those normally present in plasma with the exception of phospholipids, only a few per cent of which are recovered.

Approximately one hour after injection of labeled UFA, the radioactivity (expressed as cpm

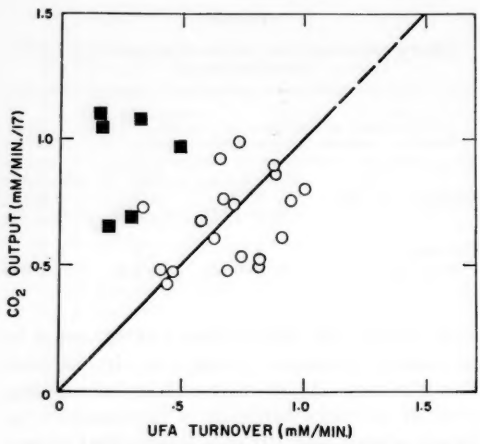

FIG. 6. COMPARISON OF THE FLUX OF UFA FROM THE PLASMA (UFA TURNOVER) WITH THE RATE OF CO_2 EXCRETION (mM $CO_2 \div 17$) IN FASTING AND CARBOHYDRATE-FED SUBJECTS

Open circles represent fasting subjects; solid squares, carbohydrate-fed subjects.

per ml. plasma) in the neutral lipid fraction exceeded that in the UFA and remained one to five times greater during the remainder of the observations. Serum taken from patients one hour after injection of linoleic and palmitic acids was extracted and passed over an ion-exchange column (21). The amount of radioactivity retained by the column (unesterified fatty acid) was comparable to that obtained in the UFA fraction by the solvent extraction procedures.

DISCUSSION

The data obtained from studies with labeled UFA described here may be combined with other available information to develop a hypothetical scheme for plasma UFA metabolism. Such a scheme can be described at the present time only in terms of a greatly simplified system of "compartments" (Figure 7). A similar hypothesis has been advanced by Dole (9).

Let A (Figure 7) represent the sources of plasma UFA. The evidence indicating that adipose tissue triglycerides probably represent the major source of plasma UFA includes: *1*) the finding of negative A-V differences for (unlabeled) UFA across areas rich in adipose tissue during times when other tissues such as the heart, liver and skeletal muscle are extracting UFA from the blood (3); and, *2*) the observation that release of UFA by rat adipose tissue *in vitro* is influenced in analogous fashion by several factors which alter plasma UFA concentrations *in vivo* (22). Should plasma UFA come largely from adipose tissue, it appears that the movement of UFA from plasma back to the adipose tissue is considerably less than the forward reaction. It has been shown that UFA-C^{14} administered intravenously to rats has little tendency to enter the adipose tissue (23), and comparison of the specific activities of plasma UFA and expired $C^{14}O_2$ obtained in these experiments (Figure 3) indicates that plasma UFA is not diluted in a pool the size of the body adipose depots before oxidation. The possibility has not been excluded, however, that there may be a significant backward flux limited to only a small fraction of the adipose fatty acids.

The flux of UFA from the plasma (B, Figure 7) to extravascular sites (C, E, Figure 7) has been measured experimentally. The half-times of disappearance for the labeled fatty acids of the order of one to three minutes are comparable to those previously reported for the dog (4–6). Laurell has also recently reported comparable findings in man for the first 10 minutes after the injection of palmitic and oleic acids (8). The high fractional turnover rates, ranging from 0.27 to 0.70 minute^{-1}, imply a very rapid diffusion rate across vascular membranes. Such rates are remarkable in consideration of the extremely high association constants between albumin and fatty acids (24). It is obvious from turnover studies with labeled albumin (25) that the albumin molecule does not leave the blood at a rate comparable to the rate with which UFA molecules leave. Extravascular albumin, sites on cell membranes having an equal affinity for UFA, or both, must assist in the removal of UFA from the plasma.

A portion of the UFA leaving the plasma compartment is rapidly oxidized. The resulting carbon dioxide eventually expired after equilibrating with the body "bicarbonate pool" (D, Figure 7) presumably represents an irreversible step in UFA metabolism. In unpublished experiments we have determined that the single injection of 5 μc. of C^{14}-labeled bicarbonate is not accompanied by any

significant labeling of plasma UFA. While the extravascular site, C, where UFA is oxidized, may not yet be precisely defined, several of its features may be surmised. It is certainly intracellular, since mitochondrial enzymes are apparently essential for fatty acid oxidation, and the immediate volume in which plasma UFA is diluted (Figure 3) is greater than extracellular fluid. The representation of the site of oxidation, C, as a single compartment is an obvious oversimplification, since effective blood flow and oxidation of fatty acids are not identical for all tissue cells. The tissue distribution of injected UFA is extensive (9, 23). Dole has reported that about 90 per cent of labeled palmitate found in tissues after injection of UFA is found in triglycerides, phospholipids and cholesterol esters (9). The neutral lipid in the plasma has been shown to become radioactive rapidly in these experiments, suggesting return of labeled fatty acids, in ester form, from the extravascular "pool" in equilibrium with plasma UFA.

The evidence that linoleic acid undergoes much more recycling into plasma, seemingly without a comparable effect on its oxidation, raises the possibility that this acid may be in equilibrium with another "compartment" not concerned with oxidation, represented by E in Figure 7. The possibility that palmitic and oleic acids also are involved in similar distribution, but with less effect on recycling, is, of course, present. While the nature of E is not known, it is pertinent that the amount of radioactivity found in the nonphospholipid-containing neutral lipid fraction was not greater after linoleic acid than after palmitic or oleic acids. It is not unlikely that E, like C, is located within tissue cells and that the difference between E and C is one of function, rather than one of anatomical location. Transfer of labeled fatty acids between E and C, therefore, may or may not require the reappearance of the molecule in the plasma compartment, B.

The contribution of plasma UFA to total energy production is a problem of special interest. This contribution could be calculated if the fraction of the expired CO_2 which is derived from plasma UFA were known. The flux of fatty acids from the plasma can be calculated, and this has been found to approximate the total CO_2 carbon excretion in the fasting human (Figure 6). However, in order for plasma UFA to account for all of the CO_2 output under the conditions observed here, the flux of UFA back into the plasma would obviously have to be negligible in the steady state. The isotopic measurements showing significant recycling of the plasma UFA indicate that this is not so (Table II). For at least one acid, linoleic, which appears to be a lesser constituent of UFA under normal conditions (17) recycling is prominent. Therefore, although the flux of plasma UFA in the human fasted 16 to 24 hours (Table III) would appear to represent the equivalent of about 2,400 calories per day, an estimation of the directness with which this UFA is oxidized is required to convert plasma turnover to actual utilization. Palmitic and oleic acids have been shown to comprise nearly 50 per cent of the UFA in some fasting humans (17). Since about half of the labeled carboxyl carbon from injected palmitic or oleic acid is shown to reappear in the plasma within seven hours (Table II), it would seem that not more than half of the quantity of these two acids which leaves the plasma is directly oxidized. Hence, the flux of UFA from the plasma may be at least twice as great as the rate at which these acids are being oxidized for energy. Undoubtedly, the relationship of plasma flux to UFA utilization is a function which depends heavily upon nutritional state, exercise and other factors affecting caloric demand. A more precise estimation of the caloric contribution of plasma UFA, based on further mathematical analysis of these and similar data obtained in dogs (16), is in preparation (26). The measurement of plasma UFA metabolism in diabetes, various disorders associated with abnormal blood lipids, and certain other abnormal metabolic states will also be described subsequently.

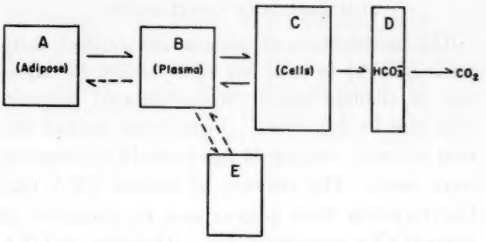

FIG. 7. HYPOTHETICAL DIAGRAM FOR PLASMA UFA METABOLISM

SUMMARY AND CONCLUSIONS

The metabolism of plasma unesterified fatty acids (UFA) in man has been studied by injection of albumin-bound carboxyl-labeled palmitic, oleic and linoleic acids. Twenty-one normal human subjects, ranging in age from 18 to 76 years, were used. The removal of labeled UFA carboxyl carbon from plasma and its excretion in expired CO_2 was measured. The flux of UFA from the plasma was found to vary with nutritional state and ranged from 0.09 to 1.5 mEq. per minute. Approximately 10 per cent of the labeled carboxyl carbon from palmitic and oleic acid was recovered as CO_2 in the first hour after injection. Calculation of isotopic flux from the plasma suggested that a large proportion of the labeled acids recycled one or more times in the plasma before oxidation, indicating that flux of UFA from the plasma may be considerably greater than the utilization of these acids for energy. Recycling of labeled linoleic acid was greater than that for oleic and palmitic acids. Labeled UFA also appeared quickly in plasma neutral lipids. A general scheme for plasma UFA metabolism is discussed.

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PERFUSION STUDIES OF THE HUMAN PLACENTA. I. EFFECT OF ESTRADIOL AND HUMAN CHORIONIC GONADOTROPIN ON CITRIC ACID METABOLISM [1, 2]

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The varied endocrine changes during human pregnancy have been intensively studied (1). The significant role of the placenta in these changes is evident from its demonstrated production of chorionic gonadotropin (2), estrogens (3) and progesterone (4). In addition, the demonstration of other hormones [corticosteroids (5, 6), corticotropin (7–9), androgens (10), relaxin (11), aldosterone (12)] in extracts of placental tissue has suggested still further endocrine functions of the placenta. However, three broad areas remain unclarified: *a*) What hormones are produced by the placenta, *b*) what are the metabolic pathways for such hormone production, and *c*) what is the interaction of placental hormones with maternal and fetal hormones and their joint contribution to the metabolic changes of pregnancy. Such basic information may have direct application to hormonal metabolism unassociated with pregnancy and also may be of clinical value in the management of disorders of pregnancy.

Organ perfusion has been a rewarding approach to the study of the metabolic functions of endocrine glands, as exemplified by the studies of Hechter and co-workers (13) with perfusion of beef adrenal glands. The extension of this general technique to the human placenta seemed to offer a promising approach to the solution of the problems outlined above. Perfusion of the whole placenta appears to be a more nearly physiologic approach to metabolic and synthetic activities of the placenta than does the study of homogenates, slices and perfused isolated cotyledons. Accordingly, we have undertaken a study of the perfusion of whole human placentas obtained immediately after full term delivery.

The normal full term placenta presents histologic (14) and metabolic (15) evidence of senescence. Therefore, it was important to demonstrate evidence of metabolic activity of the term placenta under the conditions of perfusion. Villee and Hagerman (16) have demonstrated the functional activity of enzymes of the citric acid cycle in *homogenates* of human term placenta; accordingly citrate utilization and alpha-ketoglutarate production by the perfused placenta were selected as parameters of continuing placental metabolic activity. The purpose of this paper is twofold: *1*) to report that metabolic activity of the intact term placenta persists for at least nine hours after delivery under our conditions of perfusion and *2*) to report the effect on this metabolic activity of addition of hormones to the perfusion fluid. A new action of human chorionic gonadotropin is described.

MATERIALS AND METHODS

Human placentas from normal term pregnancies were obtained two to seven minutes after vaginal delivery of the infant. The placentas were immersed in ice cold physiologic saline solution for transport to the laboratory. There the membranes were removed, the surfaces of the placenta were washed free of clots, and the organ was weighed. In preparation for the perfusion, the umbilical vein was catheterized and the larger arteries of the fetal surface of the placenta were incised. The organ was then placed in the perfusion system. The time from delivery of the baby to the beginning of perfusion did not exceed 30 minutes. The perfusion apparatus was similar to that described by Young, Prudden and Stirman (17) for liver perfusion, except that direct pulsatile perfusion was obtained by removal of the hydrostatic reservoir. The pressure as recorded proximal to the catheter averaged 220 over 80 mm. mercury.

The standard perfusing solution had the following composition in millimoles per liter: NaCl, 129.5; KCl, 2.7; $CaCl_2$, 1.8; $MgCl_2$, 0.5, NaH_2PO_4, 0.5; $NaHCO_3$, 11.9; and citric acid, 5.0. To each liter of perfusing fluid

[1] Supported by grants from the Milton Fund of Harvard University, the United States Public Health Service (RG-4802), and the Massachusetts Heart Association.

[2] A preliminary abstract was published in The Journal of Clinical Investigation, 1957, 36, 932.

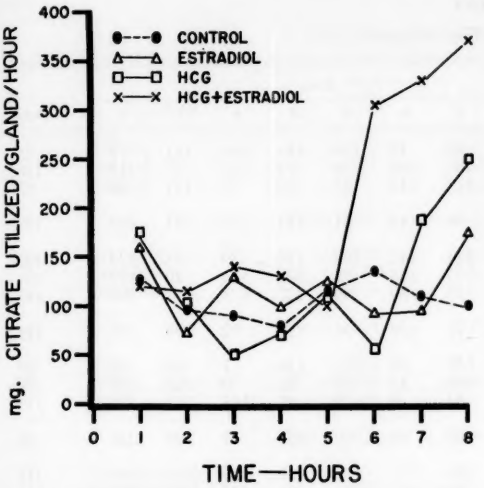

FIG. 1. THE UTILIZATION OF CITRATE BY HUMAN PLACENTAS PERFUSED WITH AND WITHOUT ADDED HORMONES

Each curve is the average of three perfusions.

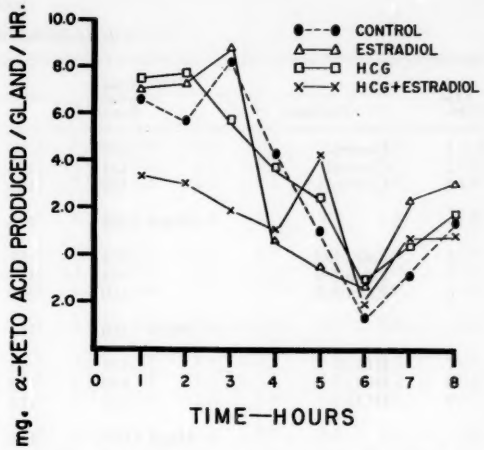

FIG. 2. THE PRODUCTION OF ALPHA-KETO ACID (MEASURED AS ALPHA-KETO GLUTARATE) BY HUMAN PLACENTAS PERFUSED WITH AND WITHOUT ADDED HORMONES

Each curve is the average of three perfusions.

were added 100,000 units of crystalline penicillin and 1.0 gram of streptomycin. In some experiments hormones were added to the standard perfusing fluid: estradiol-17 beta dissolved in 4 ml. of propylene glycol for a final concentration of 3.57×10^{-3} millimoles per liter of perfusing fluid; human chorionic gonadotropin (HCG)[3] dissolved in water for a final concentration of 20,000 International Units per liter. The perfusion fluid was passed down a Vigreux column through an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide. The pH was maintained at 7.4 and the temperature at 37 to 38° C.

An initial 20 minute perfusion was performed with one liter of standard solution to remove blood from the vascular spaces and to bring the placenta to the desired temperature. This perfusate was discarded and the system recharged with a liter of fresh perfusing fluid. Thereafter, at hourly intervals the system was drained of fluid and recharged with a liter of fresh fluid. The perfusate was recycled at an average rate of flow of 150 to 200 ml. per minute. Perfusions were carried out for eight hours. Changes in perfusing fluid volume and placenta weight were noted after each hourly run. Ten ml. aliquots of perfusate were removed for assay from the system five minutes after the start and again at the completion of each run. The aliquots were frozen and kept at -10 to -20° C. until analyzed. Citrate was determined by the method of Natelson, Pincus and Lugovoy (18) and total alpha-keto acids (calculated as alpha-keto glutaric acid) by the method of Friedemann and Haugen (19). Analyses were done in duplicate. Comparison of analyses of fresh samples with those stored in the frozen state showed no significant change. The hourly change in circulating citrate and alpha-keto acid was determined from the difference between the amounts circulating five minutes after the start of the run and at the end of the hour.

[3] Generously supplied by Ayerst Laboratories through the courtesy of Dr. John B. Jewell.

RESULTS

The effect of addition of hormones to the perfusing fluid was studied in four groups of three placentas each: *1*) standard solution, *2*) added estradiol, *3*) added HCG, and *4*) added estradiol plus HCG. Figure 1 indicates the hourly citrate utilization for each treatment group. Figure 2 indicates the hourly production of alpha-keto acid. Each curve is plotted from the means of results from three placental perfusions. The observed values are included in Tables I and II. Since the residual error term for each measurement was of similar magnitude in the different treatments,[4] combined analyses of the citrate and alpha-keto acid values, respectively, were done by the split-plot method (21). Major portions of the analysis are presented in Table III. To test the significance of differences between mean values for each treatment the mean square error term of line 2 of

[4] Bartlett's test for heterogeneity of variance (20) gave $p > 0.30$ in each instance.

TABLE I

Citrate utilization (mg. per gland) *

Expt. no.	Treatment		Gland weight (*grams*)	Hour 1	2	3	4	5	6	7	8	Mean
1	Control		639	96	80	10	129	154	165	111	17	95
2	Control		474	174	103	105	70	99	244	91	218	138
3	Control		539	106	110	152	43	90	0	131	66	87
		Mean	564	125	98	89	81	114	136	111	100	107
4	Estradiol		514	91	114	141	129	120	39	48	135	102
5	Estradiol		624	103	31	158	97	124	114	20	197	106
6	Estradiol		410	290	75	89	69	127	123	217	199	149
		Mean	516	161	73	129	98	124	92	95	177	119
7	HCG†		528	95	59	59	101	128	7	82	98	79
8	HCG		820	315	160	85	79	95	0	269	307	164
9	HCG		630	118	82	0	28	85	165	212	339	129
		Mean	659	176	100	48	69	103	57	190	248	124
10	Estradiol + HCG		467	10	153	157	186	180	420	413	412	241
11	Estradiol + HCG		651	231	88	94	101	94	143	198	281	154
12	Estradiol + HCG		628	129	104	168	107	30	351	377	422	211
		Mean	582	123	115	140	131	101	305	329	372	202

* Standard errors of mean values for three placentas of one treatment: *1*) over 8 hours, 20.9 mg. per gland per hour; *2*) at any one hour, 41.0 mg. per gland per hour.
† Human chorionic gonadotropin.

Table III was used. The significance of differences in hour to hour values was tested by the error term of line 7 of Table III. From these mean squares appropriate standard errors may be calculated. For citrate utilization and alpha-keto acid accumulation, respectively, the values are 36 and 1.14 mg. per hour for the standard error of the mean for a single placenta, 21 and 0.66 mg. per hour for the standard error of the mean for a group of three placentas, and 72 and 3.6 mg. for the standard error of a single hourly value.

Citrate utilization by the placenta was demonstrated during eight hours of perfusion with the standard solution. The values observed from hour to hour in the control group did not vary significantly ($p > 0.20$). The mean hourly utilization in this group was 107 mg. per gland. During perfusion with the standard perfusion fluid alpha-keto acid production was demonstrated. With each succeeding hour there was a highly significant ($p < 0.01$) tendency for less alpha-keto acid to be produced. During the sixth hour negative values were observed. The mean accumulation over the eight hours was 2.93 mg. per hour per gland.

When estradiol was added to the perfusion fluid there was no significant change in the amount or the pattern of citrate utilization during the eight hours of perfusion. The mean citrate utilization per hour per placenta was 119 mg. in the estradiol-treated group of three placentas. The accumulation of alpha-keto acid in the estradiol-treated group was similar to the control group and showed a similar tendency to decrease with the passage of time. The mean accumulation was 3.45 mg. per hour per gland.

In the perfusions performed with added HCG, the mean citrate utilization was 124 mg. per hour per gland, not significantly different from that of the two previous treatment groups. However, in contrast to the more uniform pattern of citrate utilization in the control and estradiol-treated groups there were significant ($p < 0.01$) hour to hour variations in the HCG-treated group without any definite trend. The citrate utilization during the eighth hour of perfusion with fluid containing HCG showed significant increase over the corresponding value for the control group ($p < 0.05$). The pattern of alpha-keto acid production in the gonadotropin group was similar to that of the

TABLE II

Alpha-keto acid accumulation (mg. per gland) *

Expt. no.	Treatment		Gland weight (*grams*)	Hour 1	2	3	4	5	6	7	8	Mean
1	Control		639	3.2	5.4	9.8	8.6	−0.9	0.7	−1.6	2.0	3.4
2	Control		474	5.6	5.1	11.9	2.5	2.3	−8.5	1.1	0.2	2.5
3	Control		579	11.0	6.5	2.9	1.5	1.6	−0.2	−2.2	1.8	2.9
		Mean	564	6.6	5.7	8.2	4.2	1.0	−2.7	−0.9	1.3	2.9
4	Estradiol		514	9.7	15.5	9.2	−1.1	−1.3	1.8	3.2	3.3	5.0
5	Estradiol		624	8.8	4.3	13.2	3.0	−1.0	−5.2	2.4	0.9	3.3
6	Estradiol		410	3.0	2.3	4.2	0.0	0.8	−0.5	1.3	4.9	2.0
		Mean	516	7.2	7.4	8.9	0.6	−0.5	−1.3	2.3	3.0	3.4
7	HCG		528	10.4	9.3	7.5	5.1	−0.4	−0.1	0.1	1.3	4.1
8	HCG		820	4.3	5.5	6.9	3.9	8.6	−4.1	−3.6	1.2	2.8
9	HCG		630	7.9	8.5	2.7	2.0	−1.0	0.8	4.6	2.6	3.5
		Mean	659	7.5	7.8	5.7	3.7	2.4	−1.1	0.4	1.7	3.5
10	Estradiol + HCG		467	11.6	5.1	1.5	1.1	2.6	−4.9	−2.1	−0.7	1.8
11	Estradiol + HCG		651	−5.1	−2.9	2.1	2.8	1.1	−2.8	4.0	1.1	0.0
12	Estradiol + HCG		628	3.6	7.0	2.2	−0.8	9.1	1.4	0.2	1.8	3.1
		Mean	582	3.4	3.1	1.9	1.0	4.3	−2.1	0.7	0.7	1.6

* Standard errors of mean values for three placentas of one treatment: *1)* over 8 hours, 0.66 mg. per gland per hour; *2)* at any one hour, 2.1 mg. per gland per hour.

previous groups. The mean of 3.50 mg. per hour per gland was not significantly different from the mean of the control or estradiol group.

Striking differences were obtained when placentas were perfused with fluid containing both estradiol and HCG. The mean citrate utilization was 202 mg. per hour per placenta, approximately twice the control value ($p < 0.02$). In addition, the hour to hour values revealed a significant regression on time ($p < 0.001$), with a marked increase in citrate utilization during the last three hours of perfusion. In the first five hours, there was no significant difference between any two groups at the same hour. Although the mean alpha-keto acid production was less in the combined treatment group (1.62 mg. per hour per gland) than in any other group, the difference was not significant. The observed hourly variation in alpha-keto acid accumulation was not significant.

A number of control experiments were performed to exclude the possibility of artifacts as the cause of the foregoing observations. To test whether citrate utilization and alpha-keto acid accumulation were the result of nonspecific changes in the perfusion fluid, 10 ml. aliquots of standard perfusing fluid which had been perfused through the placenta in the routine fashion were incubated in a shaking water bath at 37° C. and in an

TABLE III

Analysis of variance

Line	Source of variation	Degrees of freedom	Mean squares: Citrate	Mean squares: Alpha-keto acid
	Between placentas:			
1	Between treatments	3	45,190*	18.2
2	Within treatments	8	10,440	10.5
	Hourly differences:†			
3	Control	7	1,040	44.0‡
4	Estradiol	7	3,900	45.5‡
5	Chorionic gonadotropin	7	15,580‡	32.2*
6	Combined hormones	7	37,850§	12.0
7	Residual error within placentas	56	5,220	13.1

* $p < 0.05$.
† The hourly differences in the various treatment groups were analyzed separately because, as is shown by lines 3 through 7 for the citrate values, the trends were very different from treatment to treatment. The alpha-keto values were given a similar analysis in the interests of consistency.
‡ $p < 0.01$.
§ $p < 0.001$.

TABLE IV

Change in circulating potassium (mEq. per gland) *

Expt. no.	Treatment		Hour 1	2	3	4	5	6	7	8	Mean
1	Control		−0.07	+0.55	−0.46	−0.04	−0.87	+0.39	−0.13	+0.16	−0.16
2	Control		−0.84	−0.96	−0.05	+0.19	−0.01	+0.14	+0.36	−0.10	−0.06
3	Control		+1.28	−0.17	−0.69	0.0	−0.02	+0.10	+0.01	+0.10	+0.08
		Mean	+0.12	−0.19	−0.40	+0.05	−0.30	+0.21	+0.08	+0.05	−0.05
10	Estradiol + HCG		−0.53	−1.06	−0.27	−1.10	−0.35	+0.25	+0.48	+0.29	−0.29
11	Estradiol + HCG		+1.16	−0.39	+0.10	−0.37	−0.07	+0.08	0.0	−0.06	+0.06
12	Estradiol + HCG		+0.50	−0.06	−0.46	−0.07	+0.24	+0.08	+0.39	+0.06	+0.08
		Mean	+0.37	−0.50	−0.21	−0.51	−0.06	+0.14	+0.29	+0.10	−0.05

* Standard errors of mean values for three placentas of one treatment: *1)* over 8 hours, 0.097 mEq. per gland per hour; *2)* at any one hour, 0.28 mEq. per gland per hour.

atmosphere of 100 per cent oxygen for 55 minutes. Under these conditions, the concentrations of citrate and alpha-keto acid in the perfusate showed no significant or consistent change in contrast to the changes already noted during the actual placental perfusion. The average hourly change in citrate concentration during the reincubation was + 0.6 per cent with a range of − 7 to + 7 per cent. Similarly, the average hourly change in alpha-keto acid concentration during the reincubation was − 0.7 per cent.

As an index of cellular damage during perfusion the changes in circulating potassium concentration during each hourly run were recorded in six perfusions (Table IV). The potassium concentration in the perfusate was not affected by the presence or absence of hormones. In Figure 3 are depicted the average net hourly changes of circulating potassium. In the first hour of perfusion the potassium concentration tended to increase, possibly due to hemolysis of blood retained in the placenta. During the next four hours of perfusion there was a small hourly decrease in perfusate potassium followed by a slight hourly increase during the last hours.

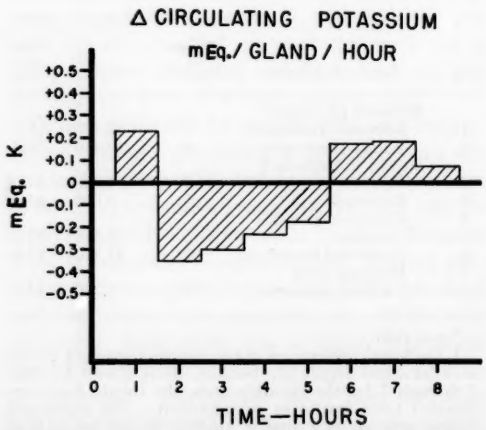

FIG. 3. CHANGES IN TOTAL CIRCULATING POTASSIUM DURING EACH HOUR OF PERFUSION

Each value is the average for six placental perfusions.

Fluoroacetate (1 mM per L.) was added to the perfusing fluid in three additional experiments (Table V). The mean values of citrate utilization were: 54 mg. for the placenta perfused with standard solution containing fluoroacetate, and 78 and 70 mg., respectively, for the two placentas perfused with standard solution containing estradiol, HCG and fluoroacetate. Using the error term of line 2, Table III, the difference between mean values observed among three placentas and one placenta, respectively, would have a standard error of 42 mg. The difference between mean values observed among three placentas and two placentas, respectively, would have a standard error of 33 mg. Thus, although the standard solution plus fluoroacetate-treated gland had one-half the citrate utilization of the corresponding group perfused without fluoroacetate, the difference was not significant ($p > 0.10$).[5] However, in the

[5] If the true effect of fluoroacetate is to reduce utilization of citrate by 50 per cent, it would be necessary to compare 10 control placentas with 10 placentas treated with fluoroacetate in order to have a 90 per cent probability of finding a significant difference at a level of $p < 0.05$.

TABLE V

Effect of fluoroacetate on citrate utilization (mg. per gland)

Expt. no.	Treatment	Gland weight (*grams*)	Hour 1	2	3	4	5	6	7	8	Mean
13	Control plus fluoroacetate	804	28	14	13	155	59	53	98	9	54
14	Estradiol HCG fluoroacetate	507	69	−34	118	102	111	68	85	111	78
15	Estradiol HCG fluoroacetate	585	40	91	62	134	11	−24	127	120	70

gland perfused with standard solution containing estradiol and HCG, the added fluoracetate completely blocked the stimulation of citrate utilization produced by these hormones ($p < 0.001$) (Figure 4). These p values are based on one-sided t tests.

The perfusate was cultured and showed no significant bacterial contamination.

DISCUSSION

These experiments demonstrate that placental metabolic activity is present for at least eight hours of perfusion of the intact gland and that this activity can be enhanced and inhibited. The response to added estradiol is not the same as noted in placental homogenates. A previously undescribed action of HCG is noted. This demonstration of a metabolically responsive gland under our conditions of perfusion establishes a firm basis for further study of the metabolic and endocrine functions of the placenta by this perfusion technique.

Fluoroacetate was used to afford additional evidence that the citrate disappearance was due to a metabolic process. Fluoroacetate, after conversion to fluorocitrate, inhibits the enzyme aconitase (22) and thus interrupts the Krebs cycle. The marked reduction of placental citrate utilization noted here in the presence of fluoroacetate indicates that such a metabolic pathway is indeed involved in the observed citrate disappearance. The failure of the added fluoroacetate to block citrate disappearance completely may have been due to failure to select an optimal concentration of fluoroacetate or to the fact that a small but constant disappearance of citrate *was* due to increasing volume of distribution during the hours of perfusion. The absence of significant weight gain by the placenta suggested that such minor distribution changes, if any, did not involve gross edema formation.

To provide an index of the degree of cellular damage and alteration in cell membrane permeability during the perfusion period, the circulating potassium concentration was determined. The relative constancy of the potassium concentration did not indicate any major shifts of potassium from the intracellular fluid. This suggested maintenance of cellular integrity during the perfusion. Moreover, failure to demonstrate changes in citrate and alpha-keto acid concentrations after reincubation of the perfused fluid indicated the absence of appropriate enzymes and cofactors for citrate metabolism in the extracellular fluid. This latter observation is in contrast to the findings of Levitz, Condon and Dancis (23) who demonstrated that after perfusion through an isolated cotyledon of human placenta, plasma gained the capacity to interconvert estradiol and estrone.

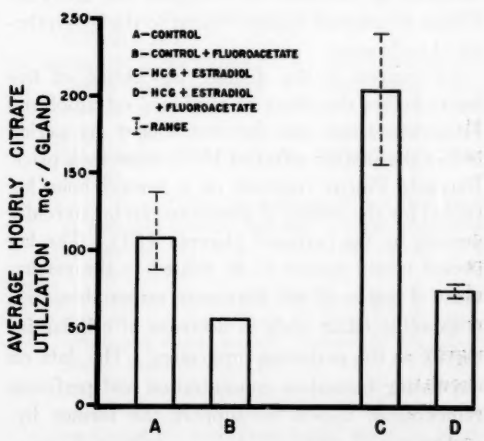

FIG. 4. THE EFFECT OF SODIUM FLUOROACETATE UPON CITRATE UTILIZATION BY PLACENTAS PERFUSED WITH STANDARD PERFUSING SOLUTION ("CONTROL") AND WITH ADDED ESTRADIOL AND HUMAN CHORIONIC GONADOTROPIN

Villee and Gordon (24–27) have studied the effect of estradiol-17β on Krebs cycle activity of human placental tissue. In a particulate-free cytoplasmic preparation this estrogen effect was manifested by increased citrate utilization and alpha-ketoglutarate production.[6] Such an estrogen effect was not demonstrated in the placenta perfusion system as here studied. Cellular permeability to estrogen may be a factor which is responsible for the discrepancy.

However, marked stimulation of citrate utilization occurred when both estradiol and HCG were added to the perfusing fluid. The ability of fluoroacetate to block completely this effect afforded confirmation that the response to hormones was a metabolic process. To a lesser degree the addition of HCG alone to the perfused placenta appeared to have an effect on citrate metabolism as judged by a significant increase of citrate utilization in the final hour of perfusion. These results are the first demonstration of such an action of HCG.

The mechanism of HCG action here is not known. A possible explanation may be that HCG increases permeability of the cell membrane to estrogen. Other loci of action of HCG might be in affecting estrogen interconversions or as a cofactor in the enzymatic reaction stimulated by estradiol. Studies on the effect of HCG on androgen secretion by the testis (29) suggest that HCG activates an enzyme system related to the biosynthesis of androgens.

Of interest is the definite lag period of five hours before the effect of combined estradiol and HCG was noted and the even longer lag period before the smaller effect of HCG alone took place. Recently Pincus reported on a several hour lag period for the effects of gonadotropin on steroidogenesis by the perfused placenta (30). The lag period would appear to be related to the mechanism of action of the hormones rather than to a nonspecific effect such as decrease of cellular integrity as the perfusion progresses. The data on circulating potassium concentration and perfusate reincubation appear to support the former hypothesis.

In our studies, alpha-keto acid accumulation was not only relatively small, but also decreased with time and showed no increase even with increased citrate utilization. This discrepancy may in part be ascribed to the lack of actual identification of the measured alpha-keto acid as alpha-keto glutarate in this study. Another explanation is that in the intact placenta the metabolic conversions of alpha-keto glutarate to glutamic acid by the transaminase and glutamic acid dehydrogenase reactions and to succinic acid by oxidative decarboxylation may not be rate limiting. The persistence of these metabolic processes in the perfused gland would thus not allow an accumulation of alpha-keto glutarate proportional to citrate utilization as noted in purified placental homogenate. The decreasing alpha-keto acid accumulation of the perfused placenta despite much larger citrate utilization may, therefore, be further evidence of continuing and widespread metabolic activity.

SUMMARY

Perfusion of the intact human placenta was undertaken to provide data concerning endocrine functions of the placenta. The presence of citrate metabolism through the eight hour perfusion confirmed the viability of the placenta when studied in this fashion.

When human chorionic gonadotropin (HCG) plus estradiol-17β were added to the perfusion fluid citrate utilization was enhanced after a five hour lag period. The addition of HCG alone appeared to stimulate citrate utilization to a lesser degree and after a longer lag period. Estradiol alone had no effect. Citrate utilization was inhibited by the addition of fluoroacetate and the response to hormonal stimulation completely blocked.

The mechanism of action of this previously undescribed effect of HCG is not known.

The differences between metabolic processes of the intact placenta and purified preparations are discussed.

ACKNOWLEDGMENT

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[6] Talalay and Williams-Ashman have recently shown (28) that the steroid activates a transhydrogenase system, thus leading to the effects noted.

The authors express their appreciation for the loyal technical assistance given by Mrs. Margaret Proctor and Miss Frances Cochios.

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STEROID FORMATION BY ADRENAL TISSUE FROM HYPERTENSIVES[1]

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The adrenal cortical hormones have been thought to play a role in the pathogenesis of hypertension for the following reasons: *1*) Hypertension is a characteristic finding in Cushing's disease; *2*) large doses of desoxycorticosterone (DCA) and salt administered to Addisonian patients produce hypertension (1); *3*) hypertensive patients who developed Addison's disease had blood pressure falls to normal limits which were restored to previous levels with DCA (*2*); and *4*) 80 per cent of the patients with severe hypertension have had significant improvement for three to seven years following 90 to 100 per cent adrenalectomy and limited sympathectomy (3). Moreover, animal experiments (4, 5) have shown that hypertension following renal artery occlusion cannot be produced in the absence of adrenal cortical steroids.

Despite these observations, altered adrenal function in hypertension has not been clearly demonstrated. While mean levels of cortical steroids in blood and urine (6–9) are not elevated in hypertensive patients, disturbances in salt and water metabolism observed in early hypertension prior to the development of renal damage by Green, Johnson, Bridges and Lehmann (10) and Braun-Menendez (11) indirectly support the idea that the pattern of adrenal corticoids is altered.

Brady (12) found that slices of canine adrenal tissue produce large quantities of steroids when incubated *in vitro* in autologous plasma and proposed the measurement of steroid formation *in vitro* as a direct assessment of the functional capacity of adrenal tissue (13). The use of adrenalectomy for the palliative management of advanced cancer (14, 15) and the treatment of severe hypertension (16) enabled us to apply this method to human adrenal tissue and to demonstrate that the amount of steroids formed by the human adrenal incubates is sufficiently large for semiquantitative estimation and identification.

In the present report we are presenting steroid analyses of adrenal tissue incubates from 3 normotensive cancer-free patients, 8 normotensive patients with carcinoma of the breast or prostate and 23 patients with severe hypertension. The differences in the steroid formation by the incubates from normotensive and hypertensive subjects and the relationship between clinical picture and steroid formation within the hypertensive group will be discussed. In addition, the steroid pattern in the incubates from hypertensive patients will be compared with that in the adrenal vein blood obtained at operation. Studies concerning the identification of the steroids will be reported elsewhere.

EXPERIMENTAL

1. Patients. The clinical data on the normotensive patients are listed in Table I. In two of the three cancer-free patients only a portion of one adrenal gland (200 to 600 mg.) was excised, while in the third a unilateral adrenalectomy was performed because of a hemorrhage occurring in the gland during the operation. No abnormalities were found by histological examination. The eight normotensive cancer patients were ambulatory and not nutritionally depleted at the time of adrenalectomy as evidenced by the satisfactory levels of serum proteins (Table I), although all had far advanced carcinoma of the breast or prostate. The adrenals from these patients were carefully examined grossly and microscopically for evidence of metastatic tumor. None was found.

Table II furnishes the clinical data of 14 of the 23 hypertensive patients studied. In these patients a com-

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[2] Charles O. Finley Fellow of American College of Surgeons.

TABLE I

Clinical data on normotensive patients

No.	Patient	Sex	Age	Height *in.*	Weight *lbs.*	Blood pressure *mm. Hg*	Diagnosis*	Duration of disease	Serum protein	Blood urea nitrogen	Urine albumin	Adrenalectomy
							A. Cancer-free patients					
1	E. O.	M	58	69	176	140/80	Sigmoid diverticulitis	10 yrs.		9	0	9/15/54
2	G. B.†	F	58	69	126	110/70	Congenital cystic dis. of kidney	6 mos.		12	trace	3/29/54
3	R. H.	M	49	67	167	130/80	Hematuria undiagnosed	1 yr.		11	trace	2/ 7/56
							B. Cancer patients‡					
1	A. E.	F	51	66	150	128/80	Breast	2 yrs.		9	0	2/17/54
2	D. B.	F	48	61.5	139	120/80	Breast	6 yrs.	7.2	13	0	12/20/54
3	L. F.	F	48	63	140	110/80	Breast	2 yrs.	6.0	12	trace	11/30/53
4	W. S.	M	62	61	135	130/60	Prostate	1–2 yrs.	6.1	13	0	4/ 8/54
5	J. B.§	M	57	64	125	132/80	Prostate	2 yrs.	5.4	16	0	1/22/54
6	G. M.§	M	58	67	173	114/68	Prostate	3 yrs.	5.4	16	1+	1/19/56
7	J. H.§	M	71	71	160	130/70	Prostate	3 yrs.	5.9	13	0	3/31/56
8	J. G.§	M	67	67	128	130/80	Prostate	4 yrs.	6.4	21	0	4/12/56

* Primary site of carcinoma in cancer cases.
† Subsequently found to have papilloma of bladder, six months hematuria prior to operation.
‡ All patients previously castrated (except those indicated by §).
§ Castrated at time of adrenalectomy.

plete chromatographic steroid analysis of the adrenal incubates was obtained. Blood pressure and adrenal weight records of the remaining eight patients in whom only the hydrocortisone values of the incubates are available are included with the latter values in Table V. The patients in Tables II and V are arranged according to ascending diastolic blood pressure. Those in Table II are divided for statistical analysis into three groups according to their clinical picture and diastolic blood pressure.

All patients were severe hypertensives who had been followed by the staff of the Hypertension Section of the Edward B. Robinette Foundation of the Hospital of the University of Pennsylvania. At the time adrenalectomy and a modified Adson type of sympathectomy were recommended they had failed to respond to several types of medical therapy and were showing progressive symptoms of the disease. All antihypertensive therapy had been stopped two weeks prior to operation. Twenty-one of the patients were on the usual hospital house diet without any restrictions. Two of the patients were in congestive heart failure as indicated in Table II and were on a salt-poor diet.

2. Preparation and extraction of the adrenal incubates. All adrenal tissue used in this study was obtained at the first stage of a bilateral adrenalectomy. No previous surgery had been done within one year in all cases studied.

Adrenal glands were dropped into an ice cooled beaker immediately after removal and transferred to a cold room (4° C.) where the periadrenal fat and capsule were removed. The glands were then weighed, a portion removed for histologic examination and the remainder sliced by means of a Stadie Riggs tissue slicer. Approximately 1.0 Gm. batches of slices were transferred to chilled 125 ml. glass-stoppered Erlenmeyer flasks equipped with gas inlet and outlet tubes in the stoppers. Ten ml. of the patient's heparinized plasma with 50,000 units of penicillin and 0.1 Gm. of streptomycin were added to each flask. The flasks were agitated in a water bath at 37.5° C. for 24 hours while a slow current of a gas containing 95 per cent oxygen and 5 per cent carbon dioxide was continuously passing through the gas space of the flasks. The shaking rate was 50 to 60 oscillations per minute through an amplitude of 4 to 5 cm. The time interval between removal of the gland and the beginning of incubation was 45 minutes or less. Five units of adrenocorticotropin (Armour) was added to selected flasks.

After incubation, the tissue and medium were quantitatively transferred to and homogenized in a Potter Elvehjem all-glass homogenizer. Five volumes of acetone was added to precipitate the protein which was separated by filtration and washed several times with additional acetone. The acetone was removed *in vacuo* in a water bath at a temperature not exceeding 45° C. The aqueous phase remaining was extracted twice with 50 ml. of ethyl acetate and twice with 50 ml. of chloroform. The combined organic extracts were washed twice with 25 ml. portions of 0.1 N sodium hydroxide and twice with 50 ml. portions of distilled water. The organic phase was dried over sodium sulfate and evaporated to dryness *in vacuo*. Adrenal vein blood was treated in a similar manner.

3. Separation of the steroids. The residue of the extracts of the adrenal incubates was subjected to paper chromatography by means of a modification of the method

TABLE II

Clinical data on 14 hypertensive patients

Group	No.	Patient	Sex	Age	Height	Weight	Blood pressure	Duration	Smith-wick group	Cardiac thoracic ratio	Electro-cardiogram	Blood urea nitro-gen	Phenol-sulfon-phthalein*	Urine albumin	Fundo-scopic grade†	Other
					in.	*lbs.*	*mm. Hg*	*yrs.*								
I	1	M. R.	M	40	69.8	141	172/80	11	1	0.49	Small. S–T elevation	28	15–25 2–65	1+	3–4	Severe diabetic
	2	C. B.‡	M	40	70.5	175	220/110	22	3	0.50	Left axis div.	13	15–20 2–60	0	4	Retinal hemorrhages
	3	M. U.	M	51	62	142	200/115	28	3	0.57	Normal	20	15–25 2–80	0	2	3 strokes; congest. heart failure
	4	A. R.	M	39	68	162	155/120	6	2	0.58	Normal	11	15–30 2–60	0	2	Headaches
	5	A. B.	F	44	66	148	190/120	15	2	0.51	Normal	10	15–35 2–75	0	2	
II	6	M. A.	F	45	64	176	210/125	17	3	0.52	T-wave changes	28	15–20 2–73	4+	3	Vertigo
	7	G. C.	M	50	72	180	220/130	0.5	3	0.56	Diphasic T-waves	18	15–25 2–50	2+	4	Headache
	8	M. M.	F	50	65.5	127	240/140	10	4	0.50	Left axis dev.	14	15–20 2–60	2+	3	Syncope
	9	F. McC.	M	50	66	180	250/140	6	4	0.54	Left axis dev.	13	15–20 2–65	1+	3	Headache
III	10	P. B.	F	25	68	115	200/146	1	4	0.46	Left axis dev.	39	15–15 2–45	3+	4	Stroke; congest. heart failure
	11	F. McL.	M	54	70.5	152	246/146	1.5	4		T-wave changes	23	15–5 2–15	1+	4	Headache
	12	J. K.	M	39	69	158	216/150	20	4	0.57	Left axis dev.	13	15–25 2–75	3+	3	Epistaxis
	13	J. D.‡	M	43	71	126	230/150	3	4	0.57	Left axis dev.	15	15–25 2–85	4+	4	Congest. heart failure
	14	T. O'C.	M	34	73	177	260/170	15	4	0.57	Left axis dev.	11	15–15 2–50	4+	4	

* Fifteen minutes and two hour per cent phenol-sulfonphthalein excretion.
† Keith Wagner classification.
‡ Not included in analyses of variance and corresponding figures.

of Burton, Zaffaroni and Keutmann (17). Three main fractions resulted. Fraction X consisted of material found on the paper in a toluene-propylene glycol system after 13 ml. of effluent had been collected per 0.5 inch width of strip. The effluent from fraction X was evaporated and rechromatographed until 3 ml. of effluent per half inch strip was collected to give fraction Y. The effluent from fraction Y was evaporated to dryness and rechromatographed in a methylcyclohexane-propylene glycol system until 17 ml. of effluent per 0.5 inch strip was collected to give fraction Z.

In all instances aliquots of the extract corresponding to approximately 0.15 Gm. of adrenal tissue were placed on each half inch width of chromatographic strip. The strips, after drying in air, were scanned at 245 mμ in a Beckman model D.U. spectrophotometer equipped with an adapter as described by Tennent, Whitla and Florey (18). The instrument was set to 100 per cent transmittance against an area of the paper strip one inch above the starting line. Representative strips were sprayed with blue tetrazolium reagent and read at 600 mμ for detection of α ketolic substances.

Absorption curves for all strips were obtained by plotting the optical density of each point against the distance from the starting line. Quantitative determinations of the amount of steroid were made by comparison of the area under the peak of absorption at 245 mμ with the area under the curve produced by known amounts of cortisol, chromatographed under identical conditions as fraction X. In nine determinations of hydrocortisone by this method, agreement with the Porter-Silber values on aliquots from the same zone was better than 95 per cent in all cases.

Results of the incubation experiments are expressed as μg. steroid formed per 24 hours per Gm. of tissue. The standard error of measurement computed from 20 duplicate determinations of adrenal incubates was plus or minus 20 μg. for the F position and plus or minus 40 μg. for the steroids of the Y and Z positions. This corresponds approximately to a reproducibility of plus or minus 5 per cent for the F position and plus or minus 8 per cent for the B-region. Recoveries of added cortisol were 95 to 105 per cent (13).

RESULTS

1. *Chromatographic pattern in adrenal incubates and adrenal vein blood*

The chromatograms shown in Figure 1 represent the absorption curves at 245 mμ from the extracts of the adrenal incubate and the adrenal vein blood of a patient. The pattern is characteristic for the entire group of patients studied. Five definite areas of absorption on the chromatograms from the adrenal incubates have been found. The first area, corresponding to material more polar than compound F, has been labeled the "before F" region. It contains several steroids not as yet identified. The second peak has been labeled as hydrocortisone on the basis of the following characteristics: The color reaction with blue tetrazolium gave a quantititative value agreeing with the degree of absorption of ultraviolet light at 245 mμ and the results of Porter-Silber determinations on selected zones. The mobility of the eluted zone in toluene-propylene-glycol and chloroform-formamide paper chromatograms as the free alcohol and as the acetate was the same as that of authentic hydrocortisone. The spectrum in sulfuric acid from 220 to 600 mμ was the same as that of the reference steroid under equivalent conditions. The characteristic green fluorescence was also shown by the eluted zones. The third peak has a mobility comparable to compound E in this system. Since several unidentified substances are present in this region, the area has been labeled the E-region.

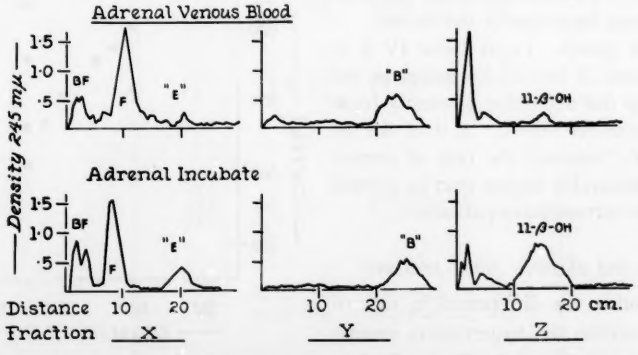

FIG. 1. CHROMATOGRAMS OF EXTRACTS OF ADRENAL VENOUS BLOOD AND ADRENAL INCUBATES

† Not included in analyses of variance and corresponding figures.

The fourth peak may be in either the Y or Z fraction and corresponds to the area in which compound B would separate. This area, designated the B-region, has recently been further separated by use of the dimethyl formamide-methylcyclohexane paper chromatography system and shown to consist of about 70 per cent corticosterone and 10 per cent Substance S [17α-hydroxydesoxycorticosterone (19)]. The remaining 20 per cent of this region has not been identified. The fifth area represents Δ^4-androstene-11β-ol-3,17-dione as has been previously reported by us (20).

The five areas representing the different steroids on the chromatograms from the adrenal vein blood are similar to those from the adrenal incubates. It is possible, however, that there are differences in the individual compounds which form the five areas since all of the substances have not been identified.

2. Steroid formation by adrenal incubates without added ACTH

a. Normotensive Group. Comparison of the values (Table III) for steroid formation by adrenal incubates from the three "normal" and eight carcinoma patients suggests that the latter do not differ greatly from those of the former, although the number of patients in the "normal" group is too small for statistical evaluation. While no comparative data on *in vitro* steroid formation of normal human adrenal tissue are available in the literature, Sweat (21) has obtained ratios of 2.8 to 3.0 for hydrocortisone to corticosterone in peripheral blood of normal subjects—a value similar to the averages of 2.62 for carcinoma and 3.09 for "normal" adrenal incubates in our series.

b) Hypertensive group. From Table IV it is seen that the pattern of steroid formation in the hypertensive group did not differ markedly from that in the normotensive series. In 9 of the 14 cases of Table IV, however, the rate of steroid formation was considerably higher than in adrenal incubates from the normotensive patients.

Steroid formation and diastolic blood pressure

To explore whether the differences in rate of steroid formation within the hypertensive groups were related to the severity of the disease, hydrocortisone formation by the incubates from the 23 patients of Tables IV and V was plotted against diastolic blood pressure. The results are illustrated in Figure 2. A significant negative correlation ($r = -0.64$; $p < 0.01$) was obtained.

In Tables II and IV the hypertensive patients have been divided into three equal sub-groups according to clinical criteria of the severity of the disease. Groups I and III comprise the least and most severe cases, respectively, at the time of operation while the intermediary cases make up Group II as recorded in Table II. In general this classification agrees well with the diastolic blood pressure arrangement, the mean diastolic pressures being 109, 134 and 156 mm. Hg for the three groups. All patients of Group III with the exception of P. B. have subsequently died. The grouping of our patients may require slight revision when a greater number of cases becomes available.

The mean hydrocortisone formation (372 μg.) by the incubates of Group I adrenal tissue was significantly greater than that (184 μg.) of the normotensive cancer patients (Tables III and VI), whereas the rate of hydrocortisone formation (184 μg.) by the Group III adrenal incubates was on the average not higher than that of the normotensive control group (the lower curve in Figure 3 and Table VI). The rate of hydrocortisone formation per unit weight of adrenal tissue thus decreased with increasing severity of hypertension.

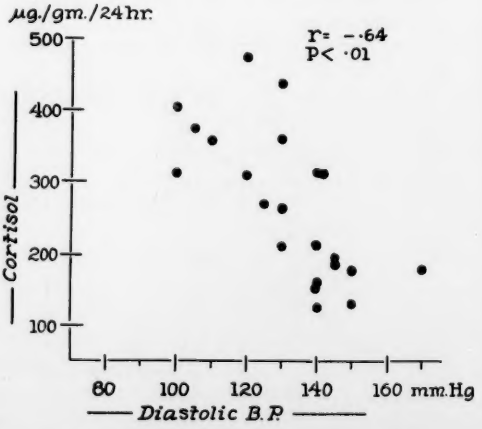

FIG. 2. RELATIONSHIP BETWEEN CORTISOL FORMATION AND DIASTOLIC BLOOD PRESSURE

TABLE III

Steroid formation by adrenal tissue from normotensive patients

No.	Patient	Adrenal weight (Gm.)	No ACTH μg./24 hrs./Gm.							With ACTH μg./24 hrs./Gm.						
			Before F	F	E	B	11-β-OH	Total	F/B	Before F	F	E	B	11-β-OH	Total	F/B
							A. Cancer-free patients									
1	E. O.		60	176	70	81	44	431	2.18							
2	G. B.	5.2	138	265	50	57	65	575	4.60	267	430	144	90	125	1,056	4.70
3	R. H.		30	222	106	90	120	573	2.50	82	410	245	150	180	1,067	2.70
	Mean	5.2	76	223	75	76	76	526	3.09	175	420	195	120	153	1,062	3.70
	S. E.*		±32.2	±25.8	±16.4	±9.85	±22.7	±54.9	±0.624							
							B. Cancer patients									
1	A. E.	4.80	66	313	108	138	124	750	2.27	138	338	64	86	267	893	3.93
2	D. B.	4.41	25	201	26	41	96	389	4.90	63	482	78	127	138	888	3.80
3	L. F.	4.60	51	124	37	117	102	431	1.07	138	345	145	272	181	1,081	1.27
4	W. S.	5.85	104	89	107	54	43	397	1.65	243	257	78	163	98	839	1.58
5	J. B.	4.30	51	155	40	41	43	330	3.78							
6	C. M.	4.57	54	267	70	67	79	537	3.99	119	429	115	327	274	1,264	1.31
7	J. H.	5.30	96	195	35	107	128	561	1.82	179	321	122	106	232	960	3.02
8	J. G.	3.80	70	131	65	91	91	448	1.44	55	176	51	155	104	541	1.14
	Mean	4.74	64	184	61	82	88	480	2.62	134	336	93	177	184	924	2.29
	S. E.	±0.223	±9.08	±26.8	±11.45	±13.88	±11.41	±47.0	±0.498	±22.6	±38.5	±13.0	±33.8	±28.1	±84.2	±0.472

* S. E. $= \sqrt{\frac{\Sigma(x_1 - \bar{x})^2}{n(n-1)}}$.

TABLE IV

Steroid formation by adrenal tissue from patients with hypertension—Cases with complete analyses

Group	No.	Patient	Adrenal weight (*Gm.*)	No ACTH *μg./24 hrs./Gm.*							With ACTH *μg./24 hrs./Gm.*						
				Before F	F	E	B	11-β-OH	Total	F/B	Before F	F	E	B	11-β-OH	Total	F/B
I	1	M. R.	6.06	68	341	99	187	74	769	1.82	94	618	88	194	99	1,093	3.19
	2	C. B.*	3.55	137	360	90	125	125	837	2.88	252	724	217	287	345	1,825	2.52
	3	M. U.	3.99	111	373	200	255	72	1,011	1.46	153	456	347	210	124	1,290	2.17
	4	A. R.	6.75	111	310	190	260	173	1,044	1.19	199	842	253	298	319	1,911	2.64
	5	A. B.	4.34	104	476	275	255	163	1,273	1.87	276	760	237	280	178	1,731	2.70
Mean	5		4.94	106	372	171	216	121	987	1.84	195	680	228	254	213	1,570	2.64
S. E.			±0.621	±11.1	±28.1	±34.5	±26.5	±21.1	±88.2	±0.287	±33.0	±66.6	±41.4	±21.5	±50.4	±160.1	±0.159
II	6	M. A.	4.80	62	270	144	218	74	768	1.24	113	466	262	298	144	1,283	1.56
	7	G. C.	5.02	123	361	165	130	170	949	2.78	141	625	328	262	199	1,555	2.39
	8	M. M.	4.22	109	314	207	318	200	1,148	0.99	177	648	352	422	285	1,884	1.54
	9	F. McC.	8.12	45	210	66	192	78	591	1.09	63	424	119	438	135	1,179	0.97
Mean	4		5.54	85	289	146	215	131	864	1.52	124	541	265	355	191	1,475	1.62
S. E.			±0.893	±18.6	±32.2	±29.6	±39.1	±32.1	±119.6	±0.456	±24.0	±56.1	±52.3	±44.0	±34.4	±157.6	±0.292
III	10	P. B.	5.81	67	196	117	140	56	576	1.40	94	230	72	252	132	780	1.74
	11	F. McL.	9.30	73	200	86	187	115	661	1.07	102	365	90	334	155	1,046	1.08
	12	J. K.	4.96	56	164	72	178	165	635	0.99	84	366	162	380	184	1,176	0.96
	13	J. D.*	6.58	57	131	51	130	110	479	1.00	79	109	96	200	79	563	0.55
	14	T. O'C.	6.74	69	175	134	180	130	688	0.97	93	462	74	336	249	1,214	1.38
Mean	5		6.68	64	173	92	163	115	608	1.09	90	306	99	300	160	956	1.14
S. E.			±0.728	±3.17	±12.5	±15.0	±17.6	±17.8	±37.1	±0.080	±4.03	±61.6	±16.4	±32.5	±28.2	±102.1	±0.200

* Omitted from analysis of variance and corresponding Figures 3, 4 and 6.

There was also a slight though less significant diminution (from 216 to 163 μg.) in the synthesis of steroids of the B region from Subgroups I to III. In all the three hypertensive groups, however, the rate of formation of B-region steroid was significantly higher than in the cancer group (*cf.* Figure 4 and Table VI). The increasing preponderance of formation of B-region steroids with increasing diastolic pressure can be most readily seen by inspecting the ratio of synthesis of hydrocortisone to synthesis of B-region steroids in Tables III and IV. This ratio declined from a mean of 2.6 in the group of normotensive patients with cancer to 1.6 in Group I of the hypertensive patients and, finally, to 1.1 in the patients with the most severe hypertension.

Steroid formation and adrenal weight

Inspection of the data in Tables IV and V reveals that the rate of hydrocortisone formation by incubates from glands weighing more than 6 Gm. was in 7 out of 9 instances below 200 μg. per Gm. per 24 hours, whereas only 2 out of 14 incubates from lighter glands displayed similarly low rates. In Figure 5 the regression of rate of hydrocortisone formation on weight of gland is depicted. The correlation proved to be significant statistically ($r = -0.65$; $p < 0.01$). Since the adrenal weights include both the weight of cortex and of medulla, it cannot be decided at present which ana-

TABLE V

Steroid formation by adrenal tissue from patients with hypertension—Cases with hydrocortisone determination only

Patient	Adrenal weight	Blood pressure	F
no.	*Gm.*	*mm. Hg*	*μg./24 hrs./Gm.*
15	3.31	200/100	312
16	5.30	198/105	405
17	4.00	200/130	440
18	3.67	245/130	210
19	5.85	270/130	263
20	5.76	210/140	314
21	10.49	220/140	112
22	7.14	240/140	127
23	6.52	240/140	156

TABLE VI

The differences in steroid formation and adrenal weight between hypertensive groups and cancer group

Group differences*	Adrenal weight (*Gm.*)	Before F (μg.)	F (μg.)	E (μg.)	B (μg.)	11-β-OH (μg.)	Total (μg.)
Without ACTH			Mean differences ± standard error†				
I—Cancer	0.12 ±0.557	41.6 ±10.45 $p < .01$	187.6 ±40.68 $p < .01$	109.8 ±30.31 $p < .01$	134.4 ±40.21 $p < .01$	33.3 ±21.91	506.4 ±90.97 $p < .01$
II—Cancer	0.80 ±0.681	20.1 ±16.29	104.4 ±44.42 $p < .05$	84.5 ±25.85 $p < .01$	132.5 ±32.29 $p < .01$	42.2 ±27.13	384.0 ±110.35
III—Cancer	1.94 ±0.629 p .02	− 0.2 ±11.92	−11.2 ±35.78	7.5 ±18.68	81.0 ±18.94 $p < .01$	26.9 ±19.99	128.0 ±73.61
With ACTH							
I—Cancer		61.2 ±38.51	344.6 ±71.90 $p < .01$	135.1 ±37.7 $p < .01$	77.2 ±44.3	28.1 ±36.61	646.3 ±166.8 $p < .01$
II—Cancer		−10.1 ±33.91	205.3 ±66.06 $p < .02$	172.0 ±41.77 $p < .01$	178.4 ±55.78 $p < .02$	5.9 ±45.50	551.5 ±161.3 $p < .01$
III—Cancer		−43.2 ±27.33	−29.0 ±68.85	4.5 ±20.69	123.8 ±48.71 $p < .05$	−25.1 ±40.99	32.1 ±108.3

* Number of experiments are 5, 4 and 5 in hypertensive groups I, II and III, respectively, 8 in cancer group without ACTH and 7 with ACTH.

† Standard error = $\pm \sqrt{\frac{\Sigma(x_i - \bar{x}_1)^2 + \Sigma(x_i - \bar{x}_2)^2}{n_1 + n_2 - 2}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$. Unless p value is recorded, difference is not significantly different from zero statistically.

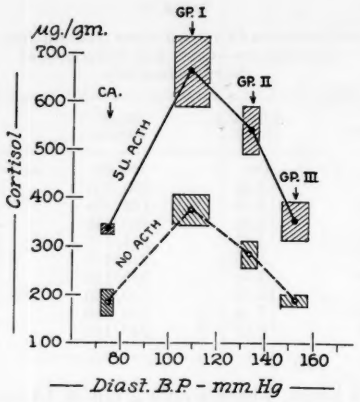

FIG. 3. CORTISOL FORMATION WITH AND WITHOUT ADDED ACTH—GROUPED DATA

The points in the center of the shaded rectangles represent the group means, the sides of the rectangles correspond to plus or minus one standard error of y and x, respectively. There are four cases in each hypertensive group as indicated in Table V. Analysis of variance of the data from the three hypertensive groups revealed a statistically highly significant association ($p < 0.01$) of cortisol formation and diastolic blood pressure and a highly significant ACTH effect ($p < 0.01$) in all three groups.

tomical constituent was responsible for the increase in weight of the whole gland.

The question arose to what extent the increased weight of the gland in severe hypertension compensated for the reduced rate of steroid formation per unit weight of adrenal tissue. To estimate the potential total capacity of steroid synthesis of the adrenal tissue present in the individual patient, the rates of steroid formation *in vitro* were multiplied by twice the weight of the gland excised. In Figure 6 the values thus computed for hydrocortisone and B-region steroid synthesis in normotensive cancer patients and the three groups of hypertensives have been plotted against diastolic blood pressure. The grand mean of total hydrocortisone output for the more severe hypertensive Groups II and III was significantly higher (931 μg.; $p < 0.05$) than that of the normotensive cancer group, whereas the rate of synthesis per unit weight of tissue had been significantly elevated only in Group I and II hypertensives. It is also seen that the total capacity to form B-region steroids did not diminish with increasing severity of the disease.

3. *Steroid formation by adrenal incubates in presence of added ACTH*

The addition of 5 units of ACTH to the incubates increased the total synthesis of steroids per Gm. of tissue by 92 per cent and 59 per cent in the normotensive cancer group and the three hypertensive groups, respectively. With the normotensive tissue the relative amounts of steroids in the different regions of the chromatograms remained essentially unchanged. Acceleration of the hydrocortisone formation by ACTH in the hypertensive groups (81 to 93 per cent) was of the same order as in the cancer group (82 per cent). However, rates of formation of E-region steroids in the hypertensive Groups I and III and of B-region steroids in Group I were not significantly stimulated by ACTH. In view of the small size of the groups, an interpretation of these comparatively slight deviations from the general response to ACTH stimulation should be postponed until confirmatory experimental evidence is available. ACTH did not alter greatly the differences between steroid formation of normotensive and hypertensive groups or within the hypertensive series. However, it appeared to augment the formation of B-region steroids in the adrenal incubates from the most severe hypertensive cases. About 31 per cent of the total steroid synthesis in

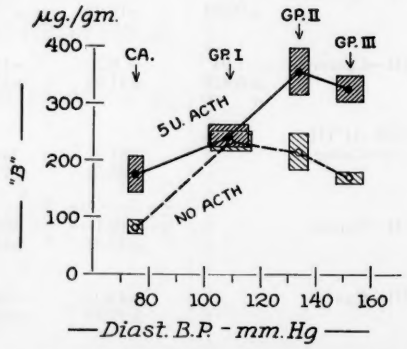

FIG. 4. FORMATION OF B-REGION STEROIDS WITH AND WITHOUT ACTH

Representation as in Figure 3. The slight decrease in steroid formation within the hypertensive groups was not significant statistically (analysis of variance).

Group III was due to steroids of the B-region as compared with 17 to 19 per cent in both Group I hypertensives and the normotensive cancer group after ACTH stimulation.

DISCUSSION

This study aimed at assessing the functional capability of normotensive and hypertensive adrenals by estimating *in vitro* steroid formation of tissue slices incubated in the autologous plasma. The close resemblance of the steroid patterns in adrenal vein blood and adrenal incubates supports the soundness of this method. The use of adrenals from the normotensive cancer patients as baseline for evaluating hypertensive alterations appeared justifiable as the data from the incubation of adrenal tissue from three normotensive cancer-free patients were in close agreement with the average of those from the cancer group.

The experiments yielded three findings of possible interpretative significance: *1*) a negative correlation between steroid formation per Gm. of tissue and diastolic blood pressure in the hypertensive group associated with a fall of the ratio of F/B steroids due to the fact that formation of B-region steroids declined significantly less with increasing diastolic blood pressure than hydrocortisone formation; *2*) a doubling of the rate of formation of all steroids, with the exception of 11β-hydroxyandrostene-3,17-dione, in the less severe hypertensive groups when compared with the normotensive group; and *3*) a positive correlation between adrenal weight and diastolic blood pressure compensating, in part, for the reduced rate of steroid formation per Gm. of tissue in advanced hypertension.

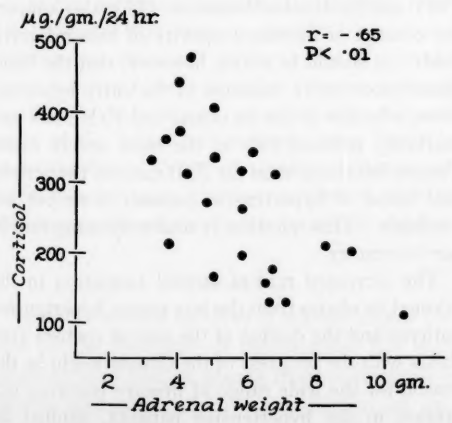

FIG. 5. RELATIONSHIP BETWEEN CORTISOL FORMATION AND ADRENAL WEIGHT

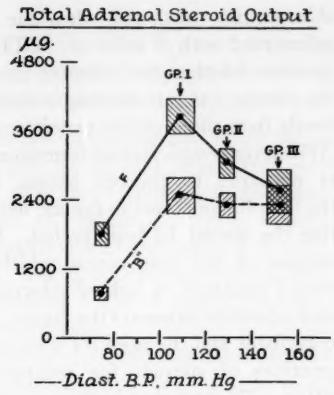

FIG. 6. TOTAL CAPACITY OF FORMATION OF CORTISOL AND B-REGION STEROIDS WITHOUT ACTH

Representation as in Figure 3. For details consult the text.

In evaluating the significance of the quantitative differences in steroid formation outlined above, consideration must be given to the fact that our assay system includes in the tissue slice and plasma two potentially independent variables. The question thus arises to what extent differences in the supply of substrates and ACTH by the individual plasmas contributed to the differences in rate of steroid synthesis.

If the difference in rate of steroid formation between the adrenal incubates from normotensive and hypertensive patients were due only to different amounts of corticotropin in the autologous plasmas used for the incubates, supplementation of the plasma medium with an excess of corticotropin should eliminate these differences. Exploratory experiments (13) have shown that the addition of 5 units of ACTH to the assay system produced maximum stimulation of steroid formation by both normotensive and hypertensive tissue. While hypertensive adrenal tissue was slightly though not significantly less stimulated than normotensive tissue by ACTH, the differences between the values from the hypertensive and normotensive groups as well as among the hypertensive groups

remained essentially unaltered when the plasma was supplemented with 5 units of ACTH.

The question whether the substrate concentration of the plasma was rate-limiting cannot be decided directly from the available experimental evidence. Whenever tested, steroid formation by the incubates appeared to proceed almost linearly during the first 12 hours and to decline but gradually during the second 12 hour period. Routine determinations of the time course which might have revealed instances of lack of substrate were not deemed advisable because of the limited amount of tissue available and the need for comparatively large quantities of steroids for separation and identification. The fact that in three exploratory experiments substitution of plasma from a normal donor for the hypertensive patient's plasma failed to alter significantly the rate of steroid formation by the incubates indicates a lack of substantial differences in substrate concentrations between the plasmas of the various groups. The fall of the F/B ratio with increasing severity of hypertension appears to be a significant observation. Since the steroids of these two regions arise from the same precursors, the shift of the ratio indicates changes in the functional capability of the adrenal cortical cells rather than differences in the supply of substrates.

Since corticosterone constitutes 70 per cent of the B-region steroids elaborated by the incubates, the fall of the F/B ratio suggests that there develops a deficiency of the 17-hydroxylase with progressing hypertension. To substantiate this interpretation it would be desirable to get quantitative information on the rate of synthesis of 17-hydroxycorticosterone (Substance S) and 18-aldocorticosterone (aldosterone), the formation of which should be affected by a deficiency in 17-hydroxylase in a similar manner as compounds F and B.

Our findings of increased adrenal weights in advanced hypertension, occasionally associated with nodularity of the cortex, corroborates the observations of earlier investigators [Rinehart, Williams and Cappeller (22); Sarason (23); Page and Corcoran (24)]. According to observations of Page and Corcoran which we can confirm, human adrenals in advanced hypertension frequently show arteriolar sclerosis. Reports in the literature (25) indicate that repeated trauma and chronic illness may cause hypertrophy of the adrenal cortex, increased weight of the glands and abnormally high rises in the plasma levels of corticosteroids following administration of ACTH. Whether incubates of adrenal slices from such glands would show alterations in the rate of steroid formation similar to those in the adrenal incubates from the enlarged glands of hypertensive patients remains an open question.

The decrease of the F/B ratio in the adrenal incubates from the patients with far advanced hypertension is of clinical interest since, besides aldosterone, corticosterone is the only salt-retaining steroid which can be detected in human adrenals (26). Perera and Pines (27), Woodbury, Rosenberg and Sayers (28), and Selye (29) suggested that decreased formation of glucocorticoids rather than over-production of mineralocorticoids might be the essential feature of hypertension, since in the dog the pathological changes produced by administration of the mineralocorticoid desoxycorticosterone could be prevented by simultaneous treatment with cortisone (28). There are several clinical reports of increased mineralocorticoid activity in essential hypertension. Genest and associates (30, 31) found increased urinary aldosterone excretion in 50 per cent of patients with advanced essential hypertension. It is of interest that aldosterone is metabolically related to corticosterone in not requiring 17-hydroxylation. The disturbances of the salt and water metabolism described by Green, Johnson, Bridges and Lehmann (10) and by Braun-Menendez (11) also support the concept of increased activity of mineralocorticoids. It should be noted, however, that the latter disturbances were manifest in the early hypertensives, whereas in the incubates the F/B ratio was markedly reduced only in the most severe cases. Precise information on the F/B ratio in the peripheral blood of hypertensive patients is as yet not available. This question is under investigation in our laboratory.

The increased rate of steroid formation in the adrenal incubates from the less severe hypertensive patients and the decline of the rate of cortisol synthesis with the progress of the disease might be the reason for the wide range of urinary corticoid excretion in the hypertensive patients, studied by other investigators. Hypercorticoiduria was observed by F. L. Selye (6), using bioassays, in 6

out of the 18 patients and by Corcoran, Page and Dustan (9) in 20 out of 46 hypertensives (formaldehydogenic steroids). The possibility of a relationship between urinary steroid excretion and diastolic blood pressure which might have explained the variability of the results, has not been taken into consideration. More direct information on the relationship between *in vitro* and *in vivo* synthesis of corticoids is provided by preliminary results from our laboratory (32) which indicated that the rate of cortisol output by adrenals *in situ*, computed from the rate of blood flow and the steroid concentration in the adrenal blood at operation prior to excision of the gland, showed a negative correlation to the diastolic blood pressure of the patient similar to that in the corresponding adrenal incubate.

Studies of the tissue metabolism *in vitro* reveal only potential cellular capabilities or deficiencies. The relevance of the *in vitro* findings for the function of the intact organ must be proved by *in vivo* observations. We have shown that the biochemical behavior of the slices from adrenal glands of hypertensive patients is consistent with some of the clinical observations on corticoid metabolism in essential hypertension. It should be re-emphasized, however, that our data on the rate of steroid formation of adrenal tissue *in vitro* concern only glands from hypertensive patients in whom the disease was progressing or had reached the malignant stage. While the alterations in the *in vitro* metabolism may be characteristic for the transition to the malignant stage of the disease, there is no evidence that they are specific for essential hypertension, since no similar series of adrenal glands from patients with other diseases producing chronic stress, except patients with advanced cancer of the breast and prostate, were available for comparison. Whether similar alterations of steroid metabolism *in vivo* are manifest in patients with various chronic diseases is being investigated.

SUMMARY

Steroid formation by slices of adrenal glands excised for the treatment of hypertension has been compared with that of glands from normotensive patients adrenalectomized for palliation of advanced cancer. The slices were incubated in the autologous plasma with and without addition of ACTH. The steroids were extracted from the incubates and separated by paper chromatography. Similar assays were done upon adrenal vein blood. The main results were:

1) On the chromatograms of all the incubates and samples of adrenal vein blood studied the steroids have been situated in five main areas designated as "before F" region, F position, E- and B-regions and Δ^4-androstene-11β-ol-3,17-dione position. The latter steroid and compound F have been identified. The B-region contains 70 per cent corticosterone.

2) The rate of steroid formation per unit weight of hypertensive gland decreases with increasing diastolic blood pressure; *i.e.*, with increasing severity of the hypertension. The decline is associated with a fall of the F/B ratio since hydrocortisone formation diminishes more than the synthesis of B-region steroids.

3) In the less advanced hypertensives the rate of formation of all steroids with the exception of Δ^4-androstene-11β-ol-3,17-dione, is about twice that in the normotensive cancer group. In advanced hypertension only B-region steroid formation is significantly augmented.

4) The weight of the adrenal gland increases with increasing diastolic blood pressure and apparently compensates, in part, for the reduction in the rate of steroid formation per unit weight of tissue.

5) The implications of the *in vitro* changes in steroid metabolism for the interpretation of the clinical findings in essential hypertension have been discussed.

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CHANGES IN TRANSMURAL CENTRAL VENOUS PRESSURE IN MAN DURING HYPERVENTILATION [1, 2]

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We demonstrated that large amounts of blood shift from the forearm veins during hyperventilation (2). If this effect were generalized in the periphery of the body the volume of blood in the central venous reservoir might increase. An increase in pressure in the central veins would be evidence to support this suggestion. The fall in peripheral venous pressure which occurs during hyperventilation (2, 3), however, implies that central venous pressure also decreases. This makes our thesis less attractive. Still, the *transmural* pressure (4), the net pressure acting to distend the central veins, *could increase* during overbreathing if the pressure surrounding the veins (intrapleural pressure) decreased more than did the pressure within them.

This study was undertaken to assess the nature and magnitude of changes in mean *transmural* central venous pressure during hyperventilation and to see whether the changes were consistent with our suggestion that blood shifts centrally during overbreathing.

METHODS

Subjects were studied in the right lateral decubitus position with the right arm extended downward through an opening in the table. Changes in central venous pressure were measured with a needle in the antecubital vein of the dependent arm according to the method of Gauer and Sieker (5). Changes in intrapleural pressure, measured as changes in esophageal pressure, were obtained by means of a small, open-ended, water-filled polyethylene tube. The tip of this tube was placed in the lower third of the esophagus. It is recognized that esophageal pressure is not identical with intrapleural pressure; however, changes in the two pressures tend to be parallel (6). Cherniak, Farhi, Armstrong and Proctor (6) observed that phasic deviations from this parallelism which did develop during spontaneous breathing resulted in intrapleural pressures which were consistently more negative than esophageal pressures. Even during intermittent positive pressure breathing the mean intrapleural pressure was more negative than the mean esophageal pressure. Thus our recorded values for *transmural* central venous pressure are less than those which actually existed.

Central venous and esophageal pressures were measured with Statham 0 to 5 cm. Hg strain gauges. The difference between these two pressures, the transmural central venous pressure, was measured with a Sanborn differential pressure transducer. The reference point for pressure was the midsternal line. End-expiratory CO_2 concentration was measured with a Liston-Becker CO_2 analyzer and ventilation was monitored with a gas meter (7). All three pressure values and CO_2 concentration were registered simultaneously with a Sanborn direct-writing oscillograph.

After resting values were obtained the subjects, male medical students, were asked to hyperventilate with maximal inspirations and passive expirations. They were prompted in order to keep ventilation above 25 L. per minute and to maintain a reduction of at least 10 mm. Hg in end-expiratory CO_2 concentration. Overbreathing was continued until values became stable. The period of overbreathing usually exceeded two minutes. This procedure was repeated in some experiments with 5 per cent CO_2 in the inspired gas. In some experiments overbreathing with 5 per cent CO_2 was repeated after the intravenous administration of 5 mg. of phentolamine methansulfonate (Regitine®).[4]

Reported mean pressures were obtained by counting squares under the curves registered by electrical integration of the output of the manometers. The sensitivity of the amplifying system was adjusted so that respiratory changes in the electrically integrated pressure curves occupied at least 2 to 3 cm. on the recording paper. In most experiments 1 cm. vertically on the recording paper was equal to 2.0 or 5.0 mm. Hg. Pressures were read to the nearest 0.5 mm. Hg.

Statistical analysis of the data was done by the methods of Fisher (8).

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TABLE I

Changes in transmural central venous pressure during air hyperventilation and during CO_2 hyperventilation

Experiment number	Esophageal pressure			Central venous pressure			Transmural central venous pressure			End-expiratory CO_2 tension			Ventilation		
	Rest air	Hyperventilation air	Hyperventilation 5% CO_2	Rest air	Hyperventilation air	Hyperventilation 5% CO_2	Rest air	Hyperventilation air	Hyperventilation 5% CO_2	Rest air	Hyperventilation air	Hyperventilation 5% CO_2	Rest air	Hyperventilation air	Hyperventilation 5% CO_2
	mm. Hg			*mm. Hg*			*mm. Hg*			*mm. Hg*			*L./min.*		
1	1.5	− 4.5		8.5	4.5		6.5	8.0							
2	1.0	− 3.0		5.5	4.0		5.0	7.5							
3	−3.0	− 4.5		9.0	8.5		11.0	12.0		41.4	28.0		11.0	20.0	
4	−4.0	− 7.0		2.5	1.0		7.0	8.0	10.0	40.7	26.2	50.3	6.1	31.5	31.2
5	−4.0	− 8.0	− 6.0	2.0	−4.0	0.0	6.0	3.5	5.5	42.6	24.0	49.4	10.5	42.0	37.0
6	−6.5	−11.0	−10.5	1.5	−2.0	1.0	8.0	9.0	11.0	40.5	22.8	45.7	6.5	32.9	31.8
7	−3.5	−12.0	−11.5	1.5	−4.0	0.0	4.5	8.0	11.0	41.7	27.7	46.7	11.7	36.8	38.3
8	−5.0	− 9.0	−10.0	3.0	0.5	3.5	7.5	8.5	12.5	44.4	28.6	49.9	5.2	32.0	35.8
9	0.0	− 6.5	− 7.5	0.0	−3.5	0.5	0.0	3.5	8.0	37.0	20.0	45.2	7.5	26.6	26.1
10	−9.5	−13.5	−14.0	1.0	−1.0	2.0	9.5	11.5	13.0	44.2	27.7	53.2	5.1	21.8	26.5
11	−5.0	− 9.5	− 9.5	4.0	0.5	3.0	8.5	9.5	12.0	46.8	28.5	51.0	15.9	34.0	27.5
12	−7.5	− 9.0	− 9.0	2.5	1.5	5.0	10.0	11.5	12.0	43.2	26.7	48.2	4.1	26.1	27.4
Experiments 5–12															
Mean difference (between resting and hyperventilation values)		− 4.7	− 4.6		−3.4	−0.1		1.4	3.9		−16.8	6.1		23.2	23.0
Standard error		0.73	0.81		0.59	0.54		0.66	0.93		0.52	0.60		1.80	2.07
Probability		<0.001	<0.001		<0.001	>0.8		<0.1	<0.01		<0.001	<0.001		<0.001	<0.001
Experiments 5–12															
Mean difference (between air and CO_2 hyperventilation values)			0.1			3.4			2.5			22.9			−0.2
Standard error			0.35			0.21			0.46			0.83			1.39
Probability			>0.7			<0.001			<0.001			<0.001			>0.8
Experiments 1–12															
Mean difference (between resting and hyperventilation values)		− 4.3			−2.9			1.4							
Standard error		0.57			0.50			0.45							
Probability		<0.001			<0.001			<0.01							

TABLE II

Changes in transmural central venous pressure during CO_2 hyperventilation before and after phentolamine administration

Experiment number	Esophageal pressure		Central venous pressure		Transmural central venous pressure		End-expiratory CO_2 tension		Ventilation	
	Before	After	Before	After	Before	After	Before	After	Before	After
	mm. Hg		*mm. Hg*		*mm. Hg*		*mm. Hg*		*L./min.*	
8	−10.0	− 8.0	3.5	1.5	12.5	8.5	49.9	49.9	35.8	40.0
9	− 7.5	− 8.5	0.5	−2.0	8.0	7.5	45.2	45.2	26.1	26.3
10	−14.0	−12.0	2.0	1.5	13.0	12.0	53.2	53.2	26.5	22.0
11	− 9.5	−10.0	3.0	−0.5	12.0	9.0	51.0	51.0	27.5	26.2
12	− 9.0	− 8.5	5.0	2.5	12.0	11.0	48.2	48.2	27.4	28.8
13			0.0	−3.5			51.3	51.3	28.0	27.8
14			3.0	0.5			47.5	47.5	23.2	23.3
15			2.5	−3.5			49.6	49.6	29.8	39.7
16			0.0	−2.5			52.2	52.2	40.5	41.7
Mean difference (Experiments 8–12)		0.6		−2.2		−1.9		0.0		0.0
Standard error		0.62		0.49		0.68		0.0		1.44
Probability		>0.3		<0.02		<0.05		>0.9		<0.9
Mean difference (Experiments 8–16)				−2.8				0.0		1.2
Standard error				0.49				0.0		1.33
Probability				<0.001				>0.9		>0.3

RESULTS

Changes in transmural central venous pressure during air hyperventilation

Mean central venous pressure fell in each of 12 experiments during air hyperventilation (Table I); the average change was 2.9 mm. Hg. Mean esophageal pressure fell in each experiment. The average change was 4.3 mm. Hg. In all but one experiment esophageal pressure fell more than central venous pressure. The difference between these two pressures, the transmural central venous pressure, *increased* by a highly significant average value of 1.4 mm. Hg during air hyperventilation ($p < 0.01$). This increase occurred in association with reduction in end-expiratory CO_2 tension which averaged 16.8 mm. Hg and increase in ventilation which averaged 23.2 L. per minute.

In Experiment 5 (Table I) mean central venous pressure decreased more than esophageal pressure during air hyperventilation. This resulted in a fall in transmural pressure. The fact that this experiment differed from the others cannot be explained. Its appearance in the group of eight subjects for which the CO_2 hyperventilation studies apply accounts for the probability value of slightly less than 10 per cent. When the entire group of 12 subjects is considered the probability of air hyperventilation not resulting in increased transmural pressure is less than one per cent.

Changes in transmural central venous pressure during CO_2 hyperventilation

Mean central venous pressure decreased in four and increased in four of eight experiments during CO_2 hyperventilation (Table I). The average change was an insignificant decrease of 0.1 mm. Hg ($p > 0.8$). Mean esophageal pressure fell in each of the eight experiments, the average change being 4.6 mm. Hg. The transmural central venous pressure decreased in one and increased in seven experiments; the average change was a highly significant increase of 3.9 mm. Hg ($p < 0.01$).

Comparison of transmural central venous pressure changes during air hyperventilation with those which occurred during CO_2 hyperventilation

Transmural central venous pressure was higher during CO_2 hyperventilation than during air hyperventilation in each of eight experiments (Table I). The difference between these two pressures was highly significant and averaged 2.5 mm. Hg ($p < 0.001$). This difference occurred because central venous pressure failed to fall appreciably during CO_2 breathing. It could not be attributed to changes in ventilation or esophageal pressure as these were the same during both air and CO_2 overbreathing.

Effect of phentolamine administration on transmural venous pressure during CO hyperventilation

Prior administration of 5.0 mg. of phentolamine methanesulfonate resulted in lower transmural central venous pressure during CO_2 hyperventilation in each of five experiments (Table II). The difference between the pressures measured before and after phentolamine was significant and averaged 1.9 mm. Hg ($p < 0.05$). This difference could not be attributed to changes in esophageal pressure, CO_2 tension or ventilation. The difference occurred because central venous pressure was lower in each instance after phentolamine ($p < 0.02$).

The fact that central venous pressure is lower during CO_2 hyperventilation following phentolamine was confirmed in four additional experiments in which transmural pressure was not measured.

DISCUSSION

Burnum, Hickam and McIntosh (9) found a substantial increase in the cardiac output of supine subjects during air hyperventilation. This was confirmed by Gleason, Berry, Mauney and McIntosh (10) who also demonstrated a similar increase in cardiac output in upright subjects during air hyperventilation. The observed increases were proportionate to the increases in heart rate so that stroke volume was maintained. Weissler, Leonard and Warren (11) found that stroke volume was not maintained in upright subjects during the tachycardia induced by atropine. They also demonstrated that cardiac output did not rise despite the marked increase in heart rate. On the basis of these observations and their own data, Gleason, Berry, Mauney and McIntosh (10) suggested that hyperventilation, unlike atropine, aids in maintaining a more adequate central blood reservoir. The suggestion that reservoir volume increases during overbreathing is consistent with the data and interpretations of these investigators. It is supported strongly (not proven) by our observations that blood shifts from the peripheral veins of part of the body (2) and that transmural central venous pressure increases with hyperventilation.

Hyperventilation could shift blood centrally by the "sucking" effect of inspiration or by the "pushing" effect of peripheral venous constriction. We demonstrated before that active venous constriction during overbreathing does push large quantities of blood from the forearm (2). The same experiments also showed that the amount of blood pushed from the forearm by venous constriction was much greater than the amount sucked from the forearm during the inspiratory efforts. These findings in the limb lead us to suggest that venous constriction may be more important generally than the aspirating effect of inspiration in moving blood into the central reservoir.

The pressure within a fluid-filled container such as the central venous system depends upon the volume within it and the tonic state of its walls. The fact that central venous pressure is greater during CO_2 than during air hyperventilation suggests to us that CO_2 overbreathing is associated with a greater central volume. The only other factor which could explain the increased pressure would be constriction of the central veins. In our opinion central venous constriction of a degree sufficient to account for a major share of the increased pressure with air or CO_2 overbreathing would be rather unlikely if the slope of the resting central venous pressure-volume curve is at all similar to that seen in the peripheral veins.

It is interesting to speculate regarding mechanisms which could explain a greater central volume with CO_2 hyperventilation. If all the peripheral veins respond as do those of the forearm (2) no more blood would be shifted from the periphery with CO_2 than with air hyperventilation. If this is true the only other possible explanation for an increased central volume is that blood is extracted from the reservoir at a slower rate (lower cardiac output) while breathing CO_2. There is some evidence for this in the report of Gleason and coworkers (10) who observed that cardiac output increased less while overbreathing CO_2 than while overbreathing air. The failure of the heart to extract blood from the reservoir as rapidly may result from the fact that the tachycardia of hyperventilation is less while breathing CO_2 than while breathing air (9). On the other hand the failure of the heart to extract blood as rapidly despite a higher transmural central venous pressure may indicate that cardiac filling is reduced because of increased diastolic ventricular tone when there

is a high concentration of CO_2 in the inspired gas.

Tenney (12) found an increased titer of sympatho-adrenal catechol amines in the blood of hypercapneic animals and cited evidence to suggest that CO_2 breathing is attended by an epinephrine-like response of the cardiovascular system in man. Our finding that prior administration of phentolamine results in a central venous pressure response during CO_2 hyperventilation which is similar to that seen with air hyperventilation is additional evidence in man to support this suggestion.

SUMMARY AND CONCLUSIONS

Intrapleural, *i.e.*, esophageal, and central venous pressure and the resultant transmural central venous pressure were measured and registered simultaneously in normal subjects at rest, during hyperventilation while breathing air, during hyperventilation while breathing 5 per cent CO_2 and during hyperventilation while breathing 5 per cent CO_2 after intravenous administration of 5 mg. of phentolamine methanesulfonate. The following observations were made:

1. Mean intrapleural pressure fell about the same amount in each kind of hyperventilation.

2. Mean central venous pressure fell regularly during air hyperventilation but remained essentialy unchanged during CO_2 hyperventilation. Mean central venous pressure, however, fell significantly during CO_2 hyperventilation after phentolamine administration.

3. The pressure distending the central veins, the transmural pressure, increased during hyperventilation in almost all experiments because intrapleural pressure fell more than central venous pressure. The transmural pressure was significantly greater during CO_2 than during air hyperventilation because central venous pressure did not fall appreciably.

4. The increase in transmural central venous pressure supports the suggestion that blood shifts centrally during hyperventilation.

5. The greater transmural central venous pressure during CO_2 than during air hyperventilation is consistent with the suggestion that blood is pumped from the central veins less rapidly. This could be attributed to the slower heart rate with CO_2 hyperventilation than with air hyperventilation. It could mean also that diastolic ventricular tone is increased with the high concentration of CO_2 in the inspired gas.

6. The central venous pressure response to CO_2 overbreathing after phentolamine administration is additional evidence that CO_2 breathing is associated with an increase in the circulating level of catechol amines.

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RELATIONSHIP BETWEEN THE AGE OF HUMAN ERYTHROCYTES AND THEIR OSMOTIC RESISTANCE: A BASIS FOR SEPARATING YOUNG AND OLD ERYTHROCYTES [1, 2]

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A study has been made of the effect of age on the susceptibility of mature human erythrocytes to hemolysis in hypotonic media. The alterations in osmotic fragility associated with red cell aging are pertinent to the relationship between cellular senescence and the maintenance of cell mambrane integrity. In addition, differences in osmotic resistance between young and old erythrocytes could provide a basis for separating the whole red cell population into fractions of differing mean cell age. A method for achieving such a separation would permit investigation of biochemical changes associated with *in vivo* aging of erythrocytes.

On the basis of experiments with red cells of dogs, Cruz, Hahn, Bale and Balfour (2) and Stewart, Stewart, Izzo and Young (3) concluded that young canine erythrocytes were *less* resistant to hypotonic lysis than older cells. Recent evidence suggests that the young erythrocytes of rabbit (4, 5), rat and (6) and man (7) are *more* resistant to hemolysis in hypotonic media than are older cells.

In the present investigation, Fe^{59} has been employed to label, *in vivo,* erythrocytes of a limited age (8–11). It has been found that graded osmotic hemolysis may achieve a separation of red cells into fractions of relatively young and old mean cell ages. The young human erythrocytes are more resistant to hemolysis in hypotonic media than older cells.

METHODS

The subjects studied included four persons in good health (Subjects LO, LA, DE and LI) and four patients with neoplastic disease. Among these patients, one had carcinoma of the breast (Subject W), two had carcinoma of the lung (Subjects Z and TE) and one had osteosarcoma (PR). The ages of these subjects ranged between 23 and 64 years and four of them were females (Subjects W, LA, LO and LI). During the entire course of study, all subjects had normal values for hemoglobin, red blood cell count, hematocrit and reticulocyte count.

Ten to 30 μc. of Fe^{59} iron globulin complex were administered intravenously to each person. At intervals up to 120 days following isotope administration, 15 to 30 ml. of heparinized venous blood were drawn for fractionation of the erythrocytes by graded osmotic hemolysis. Beginning 14 days after Fe^{59} iron administration, all subjects were given 1.2 Gm. of ferrous sulfate per day for the duration of the study.

Fractionation of erythrocytes by graded osmotic hemolysis. All blood samples were centrifuged at 1,500 × G for 10 minutes and the plasma and buffy coat removed by aspiration. The erythrocytes were washed twice with a solution of isotonic potassium chloride adjusted to pH 7.4 by addition of potassium bicarbonate and resuspended in this solution so as to provide a hematocrit of approximately 80 per cent. This procedure and all ensuing steps in the graded osmotic hemolysis were performed at 0° C. The resuspended washed erythrocytes were contaminated by fewer than 0.05 per cent leukocytes and 0.5 per cent reticulocytes.

One ml. aliquots of the resuspended washed erythrocytes were added to a series of tubes containing 5 ml. of solutions of varying concentrations of sodium chloride (usually 0.30 to 0.46 per cent sodium chloride) and a tube containing 5 ml. of distilled water. The tubes were mixed by inverting five times and allowed to stand at 0° C. for 10 minutes. The samples were centrifuged at 1,500 × G for 10 minutes and supernatant solutions removed. The sedimented unlysed erythrocytes were washed promptly with 10 volumes of the ice cold isotonic potassium chloride buffered at pH 7.4 and resuspended in approximately 2 ml. of this solution.

The concentrations of the sodium chloride solutions employed were empirically chosen to cause hemolysis of 2 plus or minus 0.5, 5 plus or minus 1, 10 plus or minus 1, 90 plus or minus 1, 95 plus or minus 1 and 98 plus or minus 0.5 per cent of the added erythrocytes. The extent of hemolysis in the water tube was taken as equal to 100 per cent. The per cent hemolysis achieved by a given concentration of sodium chloride was taken as

[1] A portion of the present data was presented before the Society for Clinical Investigation, Atlantic City, New Jersey, May, 1957, and appeared in abstract form (1).

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equal to the ratio of the hemoglobin concentration in its supernatant solution to that of the solution of water-lysed erythrocytes multiplied by 100.

Determination of Fe^{59} specific activity of hemoglobin. Hemoglobin was partially purified from the resuspended whole erythrocyte population, the water-lysed cells and the supernatant solutions and sedimented erythrocytes of the various sodium chloride tubes by a modification of the method of Drabkin (12). To one volume of each hemolysate, one-half volume of toluene was added and the mixture was thoroughly shaken until a fine emulsion was formed. After standing at 4° C. for two hours the solutions were centrifuged for 20 minutes at 4,000 × G and the water layer was removed. This "hemoglobin solution" was centrifuged at 18,000 × G for 60 minutes. A 2 ml. aliquot of the resultant clear supernatant "hemoglobin solution" was then assayed for radioactivity in a well type scintillation counter. The specific activity of these "hemoglobin solutions" was expressed as counts per minute per Gm. of hemoglobin. The relative specific activities of the "hemoglobin solutions" did not differ significantly from that of heme crystallized from these solutions (13). Hemoglobin concentrations were measured by pipetting a suitable quantity of the solution into a 3 ml. cuvette, making it up to a total volume of 2.5 ml. with 0.4 per cent ammonium hydroxide. The optical density of the solutions was determined in a spectrophotometer at 540 mμ. Duplicate hemoglobin determinations did not differ by more than plus or minus 2.0 per cent. The standard error of the counting procedure was less than plus or minus 2 per cent for most samples and less than plus or minus 5 per cent for the low activity specimens.

Iron 59 labeled hemoglobin recovery studies. It was necessary to determine whether the fractionation of the whole erythrocyte population by graded osmotic hemolysis affected the stability of the Fe^{59} labeled hemoglobin. Accordingly, the total radioactivity was measured in the "hemoglobin solutions" prepared from the supernatant solution and from the sedimented cells of the various fractions for each of the eight subjects. The sum of the counts per minute in the supernatant solution and in the sedimented cells from each fraction, expressed as per cent of the total counts per minute in the "hemoglobin solution" prepared from the whole red cell population, revealed a mean value of plus or minus one standard deviation equal to 101.5 plus or minus 4.8 per cent.

RESULTS

In the initial two studies (Subjects W and PR), washed erythrocytes prepared between 5 and 20 days following Fe^{59} administration were added to solutions of varying sodium chloride concentrations so as to give graded hemolysis between 2 and 100 per cent. In these experiments the hemoglobin Fe^{59} specific activity was lowest in the supernatant samples of the least hemolyzed fractions.

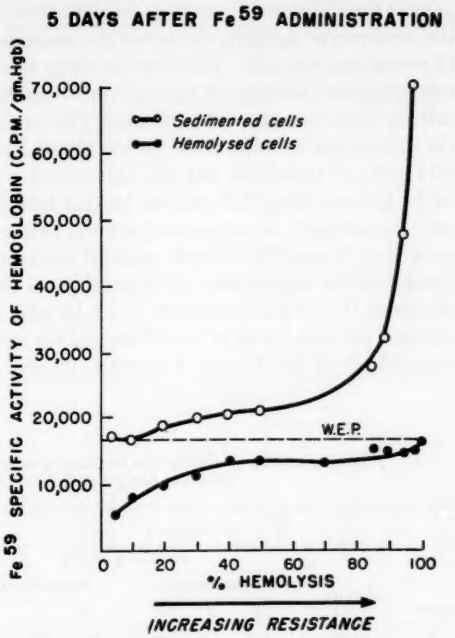

FIG. 1. Fe^{59} SPECIFIC ACTIVITY OF THE "HEMOGLOBIN SOLUTIONS" PREPARED FROM THE SUPERNATANT SOLUTIONS AND UNLYSED CELLS OBTAINED BY GRADED OSMOTIC HEMOLYSIS OF ERYTHROCYTES FIVE DAYS AFTER ISOTOPE ADMINISTRATION (SUBJECT PR)

The abbreviation W.E.P. is employed for the whole erythrocyte population.

These supernatant samples contain the hemoglobin of the least osmotically resistant cells. The highest specific activities were observed in the sedimented cells of the most hemolyzed fractions. These sedimented cells represent the most osmotically resistant erythrocytes. There were relatively small differences in specific activities of the "hemoglobin solutions" prepared from the supernatant and sedimented cells of the fractions between 20 and 80 per cent hemolysis. The data of one of these fractionations are presented in Figure 1. These findings suggested that an effective separation of the whole red cell population into fractions of relatively old and young mean cell age could be obtained by preparing the supernatant solutions of the less than 10 per cent levels of hemolysis and the unlysed cells of the more than 90 per cent levels of hemolysis. It appeared from these data that the

greater the difference in osmotic fragility between the erythrocyte samples, the better the separation of young and old cells. However, in order to obtain adequate quantities of hemoglobin for specific activity measurements, it was necessary to employ the supernatant solution of the no less than 1.5 per cent level of hemolysis and the sedimented cells of the no more than 98.5 per cent level of hemolysis. Accordingly, in subsequent studies, determinations of hemoglobin specific activity were performed on the supernatant solutions of the 2 plus or minus 0.5, 5 plus or minus 1, or 10 plus or minus 1 per cent levels of hemolysis and the sedimented cells of the 90 plus or minus 1, 95 plus or minus 1 or 98 plus or minus 0.5 per cent levels of hemolysis. For each fractionation, these data were compared to the hemoglobin Fe^{59} specific activity of the whole erythrocyte population (Table I).

During the initial 30 to 35 days after Fe^{59} administration, it was observed that, relative to the whole erythrocyte population, the specific activities were higher in the more osmotically resistant erythrocyte fractions and lower in the less osmotically resistant erythrocyte fractions. Between approximately 35 and 80 days after isotope administration, the specific activities of both the more and less osmotically resistant erythrocyte fractions were less than that of the whole erythrocyte popu-

TABLE I

Specific activity of erythrocytes fractions of varying osmotic fragility related to the specific activity of the whole erythrocyte population at intervals after administration of Fe^{59}

	Subject Z							Subject W						
		Fraction of cells‡.§							Fraction of cells					
		Most resistant			Least resistant				Most resistant			Least resistant		
Days*	Fe^{59} SA W.E.P.†	2	5	10	10	5	2	W.E.P.	2	5	10	10	5	2
5	88,000	5.70	4.40	2.07	0.17	0.07	—	116,000	3.91	—	—	0.29	—	—
10	128,000	2.68	—	1.58	0.56	0.43	—							
15	132,000	2.53	2.02	1.68	0.57	—	0.22	138,000	2.87	—	2.01	0.40	—	0.17
20														
25	138,000	1.21	1.19	1.30	—	0.45								
30														
35								140,000	—	1.36	1.16	—	—	0.31
40	140,000	—	0.65	0.75	—	0.49	0.53							
50								134,000	—	0.75	0.80	0.70	—	—
60	143,000	0.33	0.65	0.79	0.73	0.62	0.80	130,000	0.33	0.65	0.79	0.73	—	0.43
70														
80														
90	125,000	0.54	0.77	—	2.61	3.83	—	110,000	—	0.89	0.77	2.42	—	3.83
100														
110								88,000	—	—	1.04	2.32	—	2.71
120														

	Subject PR							Subject LA						
		Fraction of cells							Fraction of cells					
		Most resistant			Least resistant				Most resistant			Least resistant		
Days	W.E.P.	2	5	10	10	5	2	Fe^{59} SA W.E.P.	2	5	10	10	5	2
5	40,000	3.70	2.50	2.10	0.50	0.40	—							
10	80,000	3.50	1.90	2.20	—	0.30	—	56,000	—	2.64	2.30	0.20	0.15	—
15	88,000	—	2.00	1.60	0.40	0.20	—							
20								59,000	—	1.49	1.29	0.47	0.53	—
25	90,000	1.20	—	2.00	0.45	0.30	—							
30														
35	92,000	1.00	—	1.20	1.00	0.90	1.00							
40								62,000	—	0.72	0.80	—	0.63	—
50	84,000	—	0.30	0.60	—	0.70	—							
60														
70	89,000	0.65	0.30	0.70	0.85	0.40	0.70							
80								60,000	—	0.42	0.79	—	0.99	
90	82,000	—	—	0.70	1.70	2.80	—							
100														
110														
120	64,000	—	—	1.60	—	—	—	45,000	—	—	1.12	1.90	2.12	—

* Days after Fe^{59} administration.

† Fe^{59} specific activity of the whole erythrocyte population (W.E.P.) in counts per minute per Gm. of hemoglobin. This was taken as the average of the specific activities of the aliquot of washed whole erythrocyte population and the water-lysed erythrocytes. These specific activities differed on the average 4.2 per cent plus or minus standard deviation of 2.7 per cent.

‡ Fractions of erythrocytes are the 2 plus or minus 0.5, 5 plus or minus 1 and 10 plus or minus 1 per cent most resistant to hypotonic hemolysis and the 10 plus or minus 1, 5 plus or minus 1 and 2 plus or minus 0.5 per cent least resistant to hypotonic hemolysis.

§ The values for the ratio represents: $\frac{Fe^{59} \text{ specific activity of the fraction of erythrocytes}}{Fe^{59} \text{ specific activity of the whole erythrocyte population}}$. Where a dash (—) appears, the sample was not obtained for an analysis.

TABLE I—*Continued*

Days	Subject LO							Subject DE						
		Fraction of cells							Fraction of cells					
		Most resistant			Least resistant				Most resistant			Least resistant		
	Fe^{59} SA W.E.P.	2	5	10	10	5	2	Fe^{59} SA W.E.P.	2	5	10	10	5	2
5														
10	38,000	5.13	—	2.79	—	0.17	—							
15														
20														
25								30,000	—	—	2.37	—	0.47	—
30	41,000	—	0.41	—	—	0.30	—							
35														
40								25,000	—	0.65	0.82	0.70	0.62	—
50														
60	42,000	—	—	0.51	0.96	—	—	28,000	—	—	0.34	0.80	0.98	—
70								29,000	—	—	0.38	—	—	0.85
80														
90														
100														
110	32,000	—	—	0.78	—	2.00	—							
120														

Days	Subject LI							Subject PE						
		Fraction of cells							Fraction of cells					
		Most resistant			Least resistant				Most resistant			Least resistant		
	Fe^{59} SA W.E.P.	2	5	10	10	5	2	Fe^{59} SA W.E.P.	2	5	10	10	5	2
5								64,000	7.02	6.15	5.92	0.32	—	1.42
10								114,000	4.10	—	3.20	0.27	—	0.11
15	32,000	—	—	1.78	0.31	0.27	—	122,000	2.57	2.38	1.78	0.31	0.43	—
20								129,000	—	1.68	—	0.62	—	0.67
25														
30														
35														
40	37,000	—	—	0.84	0.54	—	—							
50														
60	36,000	—	—	0.34	0.76	—	—							
70														
80														
90														
100	41,000	—	—	1.01	1.98	—	—							
110														
120														

lation. In experiments performed at 90 days or longer following Fe^{59} administration, the specific activities of the less osmotically resistant red cells and, though to a smaller extent, the more osmotically resistant cells tended to exceed that of the whole erythrocyte population. The rise in specific activity of the more resistant (younger) erythrocyte fractions 90 days or more after isotope administration is presumably a result of the reutilization of Fe^{59}. Previous investigators have shown that while intravenously administered Fe^{59} incorporation is limited to newly forming erythrocytes, isotope released from cells as they are destroyed is available for re-incorporation into immature red cells (8, 9, 14).

DISCUSSION

The present data are consistent with the conclusions that young mature human erythrocytes are more resistant to hemolysis in hypotonic media than the average of the whole cell population, while old red cells are less resistant to osmotic hemolysis than the average of the total erythrocyte population. The method of graded osmotic hemolysis presented in this study provides a means of fractionation of the whole erythrocyte population in a manner whereby the more resistant cell fractions are progressively enriched with regard to young red cells and improverished with respect to old and middle-aged cells. The less osmotically resistant cell fractions are progressively enriched with regard to old erythrocytes and impoverished with respect to young and middle-aged cells. Among the eight subjects studied there was definite individual variation in the degree to which graded osmotic hemolysis achieved a separation of young and old red cells.

It must be emphasized that the differences in mean cell age among the fractions of erythrocytes of varying osmotic resistance are the result of a partial separation of young and old red cells. The assumption may be made that the relative

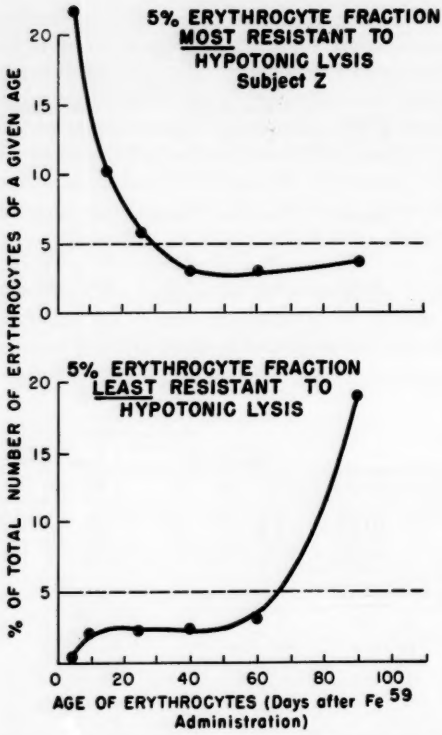

FIG. 2. THE DISTRIBUTION OF ERYTHROCYTES OF DIFFERENT AGES IN THE 5 PER CENT MOST AND LEAST OSMOTICALLY RESISTANT RED CELL FRACTIONS OF SUBJECT Z

enrichment (or impoverishment) of a particular fraction with erythrocytes of a given age is equal to the ratio of hemoglobin specific activities of the fraction to that of the whole red cell population. The validity of this assumption is based on the observations (8–11) that injected Fe^{59} will label only newly formed erythrocytes of a limited age. Such an assumption permits the estimation from the present data of the distribution of red cells of differing ages in a fraction of a given osmotic resistance. This estimation is obtained by multiplying the per cent of the total erythrocyte population in the given fraction by the ratio of the hemoglobin specific activities of the fraction to that of the whole erythrocyte population at the various time intervals following Fe^{59} administration (these ratios are presented in Table I). An example (Subject Z) of the results of the calculation of the abundance of red cells of varying ages in the 5 per cent erythrocytes most and least resistant to hemolysis in hypotonic media is given in Figure 2. Thus, in this subject, the 5 per cent most osmotically resistant cell fraction contained 22.0 per cent of all erythrocytes five days old or younger but only 3.9 per cent of all erythrocytes about 90 days of age. On the other hand, in the 5 per cent least osmotically resistant cell fraction there were 19.1 per cent of all erythrocytes about 90 days of age but only 0.4 per cent of erythrocytes five days old or younger.

The present method of graded osmotic hemolysis provides sufficiently good separation of erythrocytes into fractions of differing mean cell age to permit evaluation of the effect of age on erythrocyte enzyme activity (15). Thus, the levels of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase and phosphohexose isomerase were markedly higher in the young than old red cell fractions. Purine nucleoside phosphorylase and lactic acid dehydrogenase activities decreased relatively little with the *in vivo* aging of erythrocytes. Recently, Sterling, Greenfield and Price (6) reported that aldolase activity was higher in osmotically resistant rat erythrocytes than in the whole red cell population.

Employing a different technique for the fractionation of human erythrocytes by osmotic hemolysis, Simon and Topper (7) achieved a separation of young and old cells at 37°C. and 22°C. but not at 4°C. In addition, these authors found that at these higher temperatures, the least osmotically resistant red cell fractions were enriched with young erythrocytes, though the major portion of young cells were in the most resistant fractions. This method differed from that employed in the present study in that a single aliquot of blood was serially subjected to hemolysis in hypotonic sodium chloride solutions. The present technique was primarily developed to separate erythrocytes of varying age under conditions which would permit subsequent biochemical studies. Since certain enzymes and metabolic intermediates are less stable at higher temperatures, a study has not been made of the effect of temperatures other than 0° C. on the fractionation achieved by the present method. In the present study, with

a single exception (Subject PE, 2 per cent least resistant fraction, five days following Fe^{59} administration, Table I), no evidence was found for the enrichment of the less osmotically resistant cell fractions with young erythrocytes. The present data, however, cannot exclude the possibility that there may be red cells younger than five days which are more fragile than older cells.

Several previous investigators (16–24) have employed methods of centrifugation or sedimentation to separate red cells into populations of different mean ages. These techniques are based on the fact that young erythrocytes are less dense than old cells. The methods of centrifugation and sedimentation can provide populations of cells enriched with young and with old erythrocytes in an unlysed state, while only young red cell fractions may be obtained unlysed following separation by the technique of osmotic hemolysis. However, in experiments not requiring intact red cells, *e.g.*, studies of enzyme activity and cell free systems, graded osmotic hemolysis, compared to centrifugation, provided erythrocyte fractions with apparently greater differences in mean cell ages (15, 25).

SUMMARY

A method of graded osmotic hemolysis has been described which separates the whole erythrocyte population into fractions of differing mean cell age. This method has been validated by employing Fe^{59} labeling *in vivo* to date the age of red cells. Young mature human erythrocytes were found to be more resistant to hemolysis in hypotonic media than old cells.

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RESPONSE OF NORMAL SUBJECTS TO LARGE AMOUNTS OF ALDOSTERONE [1]

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A major effect of exogenous aldosterone in man is sodium and chloride retention and potassium excretion (1–6). Sodium retention in cases of cardiac failure, cirrhosis and nephrosis, and during sodium or fluid depletion has been associated with increased urinary excretion of this hormone (7–10). Decreased aldosterone excretion has been found with increased urinary sodium excretion during extracellular fluid volume expansion and conversely, increased aldosterone excretion has been found with decreased urinary sodium excretion in extracellular fluid volume depletion (11–13). These findings, which have established the importance of aldosterone in fluid and electrolyte metabolism, appear to conflict with the absence of marked sodium retention and edema in cases of primary aldosteronism (14, 15). It has been suggested that other adrenal steroids may modify the metabolic abnormalities of this syndrome or that an "escape" from sodium retention may occur (16–18).

The present study provides data on the effect of large amounts of aldosterone in normal subjects and indicates that following initial sodium retention, sodium excretion returns approximately to control levels despite continued aldosterone administration.

METHODS AND PROCEDURE

Studies were carried out during the autumn months on two healthy males, aged 28 and 30, who continued their usual activities but avoided vigorous exercise. Subject W. M. received *d,l*-aldosterone 21-monoacetate [4] in sesame oil, 3 mg. intramuscularly daily for 14 days, and Subject T. A. received 3 mg. daily for 4 days and 6 mg. daily for 22 days. The drug was given every eight hours. Constant diets were maintained throughout the study. These provided 3,156 calories, 128 mEq. sodium, 116 mEq. potassium and 21.3 Gm. nitrogen for Subject W. M., and 2,314 calories, 75 mEq. sodium, 84 mEq. potassium and 13.5 Gm. nitrogen for Subject T. A. Oral fluids varied between 1,750 and 2,000 ml. daily. Saliva was collected at 7 a.m. daily (19). Excretion was determined by the analysis of 24 hour urine specimens collected and stored at 5° C. and from stools pooled in three to five day periods. Control periods of six and seven days were initiated two days after the diets began. In this text electrolyte excretion during the periods of and after aldosterone administration will be compared with the average daily electrolyte excretion during these control periods. Measurements were made of sodium and potassium in serum, urine, diet, feces and saliva by flame photometry, creatinine in plasma (20) and urine (21), total nitrogen in urine, feces and diet by a micro-Kjeldahl method, urinary 17-ketosteroids (22) and 17-hydroxycorticosteroids (23), urinary osmolarity cryoscopically using a Fiske osmometer, urinary true glucose (24), arterial pH by glass electrode and urinary aldosterone (25).

RESULTS

General effects

Both subjects responded in a similar manner. Administration of aldosterone produced an initial weight increase which was more rapid in W. M. who consumed more salt (Figures 1 and 2). Weight gain slackened and ceased after an increase in body weight of 2 to 3 Kg. Subject T. A. lost an average of 0.1 Kg. per day during the control period, presumably as a result of slight caloric deficiency in the constant diet. Correcting his daily weight change by this amount indicates a pattern of weight change similar to that of Subject W. M. Arterial diastolic blood pressures increased 10 to 20 mm. mercury with maximal weight gain and remained elevated until aldosterone administration was discontinued. The only objective evidence of edema was slight periorbital

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[2] American Heart Association Research Fellow.

[3] Investigator, Howard Hughes Medical Institute.

[4] Kindly provided by Dr. Robert Gaunt, Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

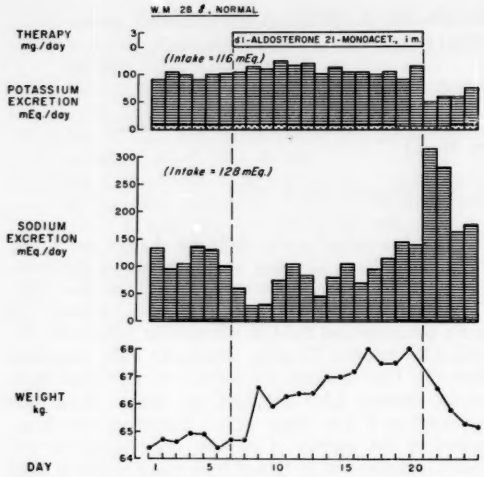

FIG. 1. THE EFFECT OF ALDOSTERONE ON WEIGHT AND SODIUM AND POTASSIUM EXCRETION IN NORMAL SUBJECT W. M. RECEIVING A CONSTANT DIET

Stool electrolyte excretion is indicated by the stippled areas and urinary electrolyte excretion by the cross-hatched areas. Where stippled areas are not seen, the stool electrolyte excretion was less than 1 mEq./day. An escape from sodium retention occurred on the fifth day of aldosterone administration.

puffiness. Headache and malaise occurred during the period of maximal sodium retention but were less severe during episodes of increased urinary volume and sodium excretion. Urinary true glucose excretion and arterial pH were unaffected.

Effect on fluid and electrolytes

Administration of aldosterone resulted in a prompt retention of sodium and an increase in potassium excretion (Tables I and II). There was no apparent relationship between sodium retention and potassium excretion: Sodium retention was greater with higher sodium intake and diminished as the treatment continued; potassium excretion was essentially similar in both subjects and always exceeded the average daily control potassium excretion except for one day in Subject W. M. Consequently, sodium retention initially greatly exceeded potassium excretion, but as treatment continued, the opposite occurred and potassium excretion exceeded sodium retention. Sodium excretion was never less than 10 mEq. per 24 hours despite the large amount of aldosterone, and was smaller in Subject T. A. who received more aldosterone and less sodium than Subject W. M.

Despite continued aldosterone administration, sodium excretion suddenly increased in both subjects. In Subject W. M. this occurred on the fifth day of aldosterone administration, following cumulative weight gain of 1.5 Kg., and cumulative sodium retention of 270 mEq., as compared to the average daily sodium excretion during the control period. In T. A. this occurred on the sixteenth day, following weight gain of 3 Kg. and sodium retention of 536 mEq. Urinary sodium excretion then assumed a cyclical pattern with peaks of excretion and retention every 4 to 5 days. Subject W. M., during the remaining 9 days of aldosterone administration, retained an additional 181 mEq. of sodium but during two days excreted more sodium than during the average control day. Subject T. A., during the remaining 10 days, excreted 73 mEq. of sodium in excess of the expected control excretion for that period. As compared to the average daily control excretion, cumulative electrolyte changes during the entire period of aldosterone administration for W. M. were plus 16.0 Gm. nitrogen, plus 451 mEq. sodium, and minus 211 mEq. potassium[5]. For T. A. they were plus 0.3 Gm. nitrogen, plus 463 mEq. sodium and minus 510 mEq. potassium.[5] Prompt sodium excretion and potassium retention followed omission of aldosterone.

The salivary sodium: potassium ratio was reduced during the treatment period, chiefly due to decreased sodium concentration; there was no change when urinary sodium excretion increased.

There was no significant change in serum sodium or potassium concentrations. Stool electrolyte changes suggest reduced intestinal sodium loss in W. M. and increased potassium loss in T. A.

Effect on urinary steroid excretion

17-Ketosteroid and 17-hydroxycorticosteroid excretion was essentially unchanged during the entire study. Urinary excretion of aldosterone averaged 200 μg. and 100 μg. per 24 hours when 6 and 3 mg., respectively, of the *d,l*-aldosterone 21-monoacetate were administered.

[5] Potassium was corrected for nitrogen with an assumed K/N ratio of 2.7.

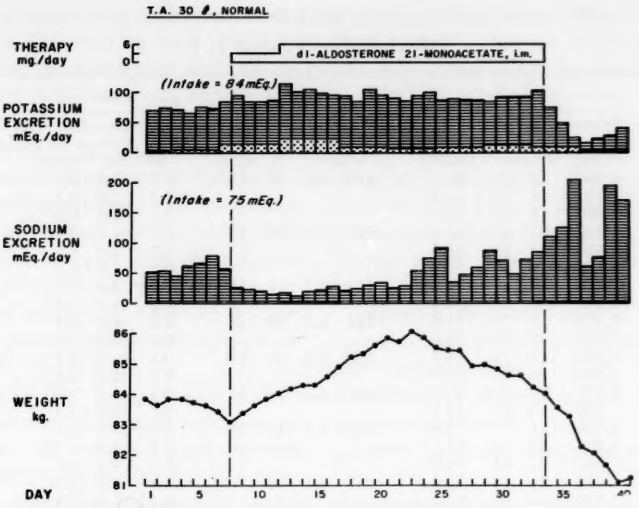

FIG. 2. THE EFFECT OF ALDOSTERONE ON WEIGHT AND SODIUM AND POTASSIUM EXCRETION IN NORMAL SUBJECT T. A. RECEIVING A CONSTANT DIET

Stool electrolyte excretion is indicated by the stippled areas and urinary electrolyte excretion by the cross-hatched areas. Where stippled areas are not seen, the stool electrolyte excretion was less than 1 mEq./day. An escape from sodium retention occurred on the sixteenth day of aldosterone administration.

TABLE I

Metabolic data on Subject W. M.

Day	Body wt.	Urine				Blood				Stool			Saliva		Therapy Aldosterone
		Volume	Na	K	N	Na	K	Hct.*	BUN†	Na	K	N	Na	K	
	Kg.	*ml./day*	*mEq./day*		*Gm./day*	*mEq./L.*		*%*	*mg. %*	*mEq./day*		*Gm./day*	*mEq./L.*		*mg./day*
1	64.43	3,440	133	81	16.0	138	4.1	50.5	15	0.4	8.0	1.5	17	21	0
2	64.66	2,800	93	97	15.2					0.4	8.0	1.5	22	23	0
3	64.55	2,470	102	92	18.2	138	4.3	49.5	16	1.5	7.5	1.5	21	24	0
4	64.89	3,380	135	81	19.7					1.5	7.5	1.5	19	22	0
5	64.89	3,520	130	93	18.6					1.5	7.5	1.5	21	22	0
6	64.43	2,920	99	94	17.9	137	4.3	50.5	15	1.5	7.5	1.5	20	23	0
7	64.66	2,580	60	96	20.5					0.3	9.7	1.6	19	23	3
8	64.66	2,860	28	106	17.4	136	3.9	49.0	14	0.3	9.7	1.6	16	24	3
9	66.59	2,800	31	101	17.1					0.3	9.7	1.6	9	25	3
10	65.91	3,320	75	116	16.9	140	4.0	45.0	13	0.3	9.7	1.6	12	27	3
11	66.25	3,840	104	109	18.2					0.2	7.9	1.7	13	27	3
12	66.36	3,340	83	113	17.4					0.2	7.9	1.7	13	24	3
13	66.36	2,740	46	95	14.9	141	4.0	45.5	11	0.2	7.9	1.7	13	27	3
14	67.05	3,560	79	106	15.8					0.2	7.9	1.7	9	26	3
15	67.05	3,220	104	99	14.6	142	3.8	45.5	11	0.3	8.0	1.9	12	26	3
16	67.16	3,720	71	99	13.4					0.3	8.0	1.9	14	24	3
17	67.95	2,730	94	94	16.2	144	3.9	45.0	11	0.3	8.0	1.9	9	25	3
18	65.50	3,440	115	98	14.8					0.3	8.0	1.9	10	27	3
19	67.50	3,280	143	83	15.8	142	3.8	44.0	12	1.2	6.0	1.4	10	28	3
20	67.95	3,680	138	109	15.1					1.2	6.0	1.4	9	26	3
21		4,560	314	46	17.6					1.2	6.0	1.4	9	26	0
22	66.59	4,040	280	54	15.6	144	4.0	46.0	13	1.2	6.0	1.4	14	22	0
23	65.80	2,970	162	53	15.1					1.7	8.4	1.2	18	23	0
24	65.34	2,920	175	69	20.1	141	5.2	51.0	15	1.7	8.4	1.2	18	22	0
25	65.23														

* Hematocrit.
† Blood urea nitrogen.

TABLE II

Metabolic data on Subject T. A.

Day	Body wt.	Urine				Blood				Stool			Saliva		Therapy Aldosterone
		Volume	Na	K	N	Na	K	Hct.*	BUN†	Na	K	N	Na	K	
	Kg.	*ml./day*	*mEq./day*		*Gm./day*	*mEq./L.*		*%*	*mg. %*	*mEq./day*		*Gm./day*	*mEq./L.*		*mg./day*
1	83.86	1,260	52	67	10.7	139	4.0	46	15	0.3	3.0	0.5	30	18	0
2	83.63	1,120	54	70	16.3					0.3	3.0	0.5	39	18	0
3	83.86	1,080	45	68	14.1					0.2	4.0	0.5	39	18	0
4	83.86	1,140	63	61	15.6	140	3.9	47	13	0.2	4.0	0.5	31	19	0
5	83.75	1,160	66	73	15.0					0.2	4.0	0.5	35	18	0
6	83.63	1,180	79	69	14.6					0.2	4.0	0.5	33	18	0
7	83.45	978	57	81	15.3	138	4.3	43	15	0.2	4.0	0.5	38	20	0
8	83.09	1,190	26	81	14.4					0.2	14.0	2.1	39	20	3
9	83.40	1,070	23	70	14.6	142	3.7	44	14	0.2	14.0	2.1	36	20	3
10	83.63	790	21	71	14.0					0.2	14.0	2.1	32	21	3
11	83.86	1,120	16	73	14.7	138	3.6	44	15	0.2	14.0	2.1	26	22	3
12	84.09	1,100	16	92	14.5					0.7	24.0	2.2	25	21	6
13	84.20	1,020	12	77	13.7	138	4.0	43	12	0.7	24.0	2.2	19	23	6
14	84.30	980	18	82	14.8					0.7	24.0	2.2	23	22	6
15	84.32	1,120	21	74	13.9	137	3.5	43	10	0.7	24.0	2.2	18	21	6
16	84.60	1,100	27	68	14.7					0.7	24.0	2.2	20	23	6
17	84.90	960	22	85	12.8					0.1	7.0	0.9	21	22	6
18	85.22	920	24	76	12.0	136	3.4	42	7	0.1	7.0	0.9	20	23	6
19	85.34	1,150	30	99	13.2					0.1	7.0	0.9	22	23	6
20	85.60	1,030	34	89	12.8	137	3.2	41	9	0.1	7.0	0.9	18	23	6
21	85.86	1,160	26	74	12.9					0.1	6.0	1.0	20	23	6
22	85.72	940	28	78	13.3	143	3.3	40	11	0.1	6.0	1.0	21	23	6
23	86.09	1,740	54	89	12.5					0.1	6.0	1.0	21	21	6
24	85.86	1,760	74	93	12.8					0.1	6.0	1.0	21	20	6
25	85.56	1,280	91	77	12.0	144	3.5	40	8	0.3	10.0	1.3	21	23	6
26	85.45	1,600	34	81	14.1					0.3	10.0	1.3	23	23	6
27	85.45	1,060	47	78	13.3	144	3.4	40	10	0.3	10.0	1.3	17	23	6
28	84.95	1,140	58	78	13.6					0.3	10.0	1.3	17	22	6
29	85.00	1,520	87	72	13.1	144	3.2	41	11	0.3	13.0	0.9	20	25	6
30	84.88	1,260	71	80	13.2					0.3	13.0	0.9	18	24	6
31	84.64	1,340	47	69	14.0					0.3	13.0	0.9	17	23	6
32	84.65	1,420	73	79	14.0	139	4.0	41	8	0.3	13.0	0.9	12	24	6
33	84.22	1,560	85	95	14.0					1.4	9.0	1.6	14	23	6
34	84.04	1,685	109	66	14.2	140	3.0	41	8	1.4	9.0	1.6	17	24	0
35	85.59	1,440	125	39	13.7					1.4	9.0	1.6	23	22	0
36	85.30	2,220	203	16	13.8	144	3.0	41	10	1.4	9.0	1.6	21	22	0
37	82.27	1,220	59	14	14.5					0.6	3.0	0.6	27	17	0
38	82.09	1,940	75	20	13.5					0.6	3.0	0.6	29	17	0
39	81.64	1,780	193	26	14.2	137	3.6	46	13	0.6	3.0	0.6	29	18	0
40	81.09	1,540	167	38	16.5					0.6	3.0	0.6	40	18	0
41	81.25														

* Hematocrit.
† Blood urea nitrogen.

Effect on renal function

There were no demonstrable differences in the 24 hour creatinine clearances between the control and treatment periods. Urine concentration during Pitressin® stimulation on the days before and after aldosterone administration was greater than 1,000 mOsm. per Kg. of water in both subjects.

DISCUSSION

Long term administration of desoxycorticosterone acetate (DCA) to human subjects has been shown to produce only transient retention of sodium (26–28), but a constant increase in exchangeable sodium of 500 to 700 mEq.. (29). The response to large amounts of aldosterone observed in this study appears to be similar. In two normal subjects maximal weight gains did not exceed 3.5 and 3.0 Kg., sodium and water retention occurred for only a few days, and significant edema did not appear, despite continued administration of 3 and 6 mg. of *d,l*-aldosterone 21-monoacetate daily. The data imply that fluid retention in excess of 3 to 5 L. is not to be expected with primary aldosteronism unless other disease processes in-

terfere with the mechanism responsible for sodium and water excretion following an initial period of retention. One may postulate that such an abnormality may exist in patients with cardiac failure, cirrhosis and nephrosis when these disease states are associated with significant edema. Viewed in this manner, factors other than aldosterone assume great importance in the production of edematous states. When primary aldosteronism occurs in conjunction with these other disease processes prominent fluid retention may be expected to occur. A recent case report of primary aldosteronism and edema invites such an explanation (30).

The mechanism of this "escape" from sodium retention during continued administration of aldosterone or other salt retaining corticosteroids is not completely understood. Changes in volume or electrolyte concentration relationships, acting on the kidneys directly or through hormonal or neural pathways, must be considered. Previous studies indicate that natriuresis during administration of DCA does not occur when sodium is restricted (28, 29). In this study, the renal response to aldosterone was apparently dependent upon either fluid volume increase or sodium retention rather than duration of treatment, as shown by the more rapid return to sodium balance with greater sodium intake. That volume is the more important of the two is indicated by evidence that sodium excretion can be induced despite sodium restriction by extracellular fluid volume expansion with Pitressin® and water (12). The method by which this volume expansion results in increased sodium excretion is unknown. In these experimental subjects it obviously cannot be attributed to inadequate adrenal aldosterone production. A change in the metabolism of aldosterone with increased or more rapid hormone inactivation seems unlikely in view of the constant effect on urinary potassium and salivary sodium excretion. Continued depression of the salivary sodium: potassium ratio during the period of increased urinary sodium excretion demonstrates that other effects of aldosterone remain unchanged. This may represent differences in tissue response to the hormone or the effect of other factors acting on the kidney. Secretion of an adrenal sodium excreting hormone or, in the case of primary aldosteronism, of other corticosteroids which interfere with sodium retention may be implicated (16, 31–33). The role of the kidney in this mechanism is still a subject of debate. Increased glomerular filtration rate during volume expansion has been claimed and disputed, and the significance of any elevation is uncertain. Changes in renal tubular function have been postulated but adequate experimental evidence of such is not available. Several recent reviews are devoted to this subject (34–36).

Several authors have pointed out the seemingly "paradoxical" sodium excretion in cases of primary aldosteronism (14, 15, 37, 38). During administration of either ACTH or hydrocortisone, sodium retention occurred for one to three days, followed by sharp natriuresis while the hormone was being given. One may postulate that this phenomenon is based on the same mechanism responsible for the sodium excretion seen in this study. Patients who have already achieved salt and water balance despite primary aldosteronism, when given adrenocortical hormone, will either not retain sodium or will do so for only one or two days before negative sodium balance compensating for this additional sodium retention occurs. The return to sodium and water balance occurs more quickly than seen in this study because maximal or near maximal fluid retention is already present and the stage is set for sodium excretion. Evidence in favor of this concept is found in the natriuresis following acute expansion of body fluids with saline in prehydrated subjects (39, 40), patients with Cushing's syndrome (41, 42), and subjects pretreated with ACTH or corticosteroids (43, 44).

Expansion of extracellular fluid volume with intravenous saline or Pitressin® and water will lead to diminished aldosterone and increased sodium excretion (11–13, 45); that sodium excretion results from diminished aldosterone has been suggested (11–13). In addition, Wrong has postulated that the 6 to 12 hour delay in sodium diuresis following body fluid expansion may be due to slow disappearance of aldosterone from the circulation (46). This study indicates that these two phenomena need not be related; sodium excretion during volume loading occurs despite continued aldosterone administration.

SUMMARY

1. Two normal subjects were given 3 and 6 mg. of *d,l*-aldosterone 21-monoacetate in sesame oil intramuscularly while on a constant diet.

2. Following an initial period of weight gain and sodium retention, weight gain ceased and sodium excretion returned approximately to control levels, despite continued administration of the hormone.

3. Increased potassium excretion occurred throughout the period of aldosterone administration.

4. These findings are consistent with the lack of edema in primary aldosteronism and demonstrate that sodium excretion during volume loading need not be related to diminished aldosterone secretion.

5. It is postulated that the "paradoxical" sodium excretion seen in cases of primary aldosteronism occurs as a result of the "escape" phenomenon.

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LIPOLYTIC ACTION OF CORTICOTROPIN ON RAT ADIPOSE TISSUE *IN VITRO* [1, 2]

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It has been reported from this laboratory that under the proper experimental conditions corticotropin is capable of modifying metabolic processes in the mouse and rat through mechanisms other than by stimulating the secretion of steroid hormones by the adrenal cortex (1–5). Similar observations have been made by Astwood (6). The metabolic responses to adrenocorticotropic hormone (ACTH) in both intact and adrenalectomized animals bear a striking similarity to those induced by growth hormone (Table I) and some have also been elicited with the thyroid stimulating hormone (2, 3). Available data so far do not support the notion that these common metabolic actions are due to impurity of the hormone preparations used, with either growth hormone or some as yet unidentified "metabolic" hormone being the common contaminant.

Elsewhere the hypothesis has been proposed that the sharing of a variety of metabolic activities by pituitary hormones may simply reflect the fact that the tropic hormones serve as growth and metabolic hormones for their target glands in addition to being specific stimulators of hormone synthesis and release (3). Since the pathways for growth and general metabolism presumably are largely the same in almost all tissues it should not be surprising to find that the tropic hormones might be capable of influencing metabolism in tissues other than their target glands. The specificity of the tropic hormones might then relate either to a greater affinity of the hormone to the target gland or, alternatively, to a greater sensitivity of the gland to the hormone. In the case of ACTH, which promotes steroidogenesis by the adrenal cortex and hence must have a potent influence on lipid metabolism in the gland, it has seemed reasonable to search for extra-adrenal influences of the hormone on pathways of lipid metabolism which might be considered analogous to those occurring in the adrenal cortex.

One of the impressive effects of ACTH is its ability to cause a loss of lipid from the adrenal cortex (7). The same hormone has been found capable of inducing ketosis, fatty liver and a loss of extractable lipid from adipose tissue depots in mice and rats (2, 4, 6, 8). The purpose of the present study was to determine whether ACTH might influence the metabolism of adipose tissue *in vitro*. It was found that the ACTH was highly effective in stimulating the hydrolysis of neutral fat and release of nonesterified fatty acids when incubated with rat adipose tissue in plasma and other suitable media.

METHODS AND MATERIALS

Twenty to 60 mg. portions of epididymal adipose tissue were removed from 250 to 350 Gm. Wistar rats, which had been fasted overnight and anesthetized with sodium pentobarbital injected intraperitoneally. The adipose tissue was weighed on a Roller-Smith torsion balance and then incubated in 1 ml. of pooled rat plasma at 36° C. in a Dubnoff shaking incubator. Heparinized plasma was obtained by exsanguinating 350 to 450 Gm. male rats via the abdominal aorta and was used immediately or after refrigeration. When compared with a few pilot studies done with serum, heparinized plasma was found to yield comparable results and was therefore used for convenience. The gas phase was air or nitrogen. The 10 ml. Erlenmeyer flasks used for incubation were left unstoppered. Evaporation was retarded by enclosing the flasks in a humidifying hood.

Plasma fatty acids were determined on 0.4 ml. aliquots of plasma by the method of Dole (9) immediately and three hours after the addition of the hormone solution or an equal volume of distilled water for the control.

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TABLE I

Extra-adrenal metabolic activities common to ACTH and growth hormone

Mobilize Fat from Depots (8)
Increase Liver Fat (2, 6)
Induce Ketosis (4, 6)
Depress R. Q. (6)
Induce Hypoglycemia (5, 6)
Increase Glucose Tolerance (5)
Increase Adipose Tissue Glycogen (Insulinotropic) (5)
Increase Cardiac Glycogen (6)
Enhance Insulin Action on Glucose Uptake by Rat Diaphragm *in Vitro* (16)
Decrease Urea Formation from Infused Amino Acids in Nephrectomized Rats (3)

Tissue fatty acids were determined, after rinsing the adipose tissue samples in distilled water, by macerating them with a glass rod in the same extraction mixture used for plasma and by then following the same procedure as in the plasma method.

The following hormones were used:

Corticotropin (see Reference 4 for further description of these samples). *1.* Armour Laboratories. Porcine corticotropin "A," lot No. 980-001-1, 125 U.S.P. units per mg. Protein ACTH, "Acthar-A®," lot R, J 17409, 1 unit per mg. *2.* Lederle Laboratories, American Cyanamid Company. α_1-α_2 pool—lot No. S-1079-28; 80-100 U.S.P. units per mg. γ pool—lot No. S-1079-45; 80-100 U.S.P. units per mg. δ-1—lot No. S-1079-24; 10 U.S.P. units per mg. $\beta P_s(3)$—lot No. S-1892-34 No. 225. Peptide from pepsin digestion of ACTH; 80-100 U.S.P. units per mg. C-10—Peptide from chymotrypsin digestion of ACTH. No ACTH activity. *3.* Wilson Laboratories. Oxycellulose adsorbed ACTH, lot No. 102621; approximately 140 U.S.P. units per mg.

These preparations were dissolved in distilled, deionized water prior to use.

Melanophore stimulating hormone (*MSH*). *1.* α-MSH-35-B, from Dr. S. Steelman; *2.* β-MSH-CCD-4, from Drs. I. Geschwind and C. H. Li.

Each preparation of MSH was dissolved in distilled, deionized water just prior to use.

Growth hormone (*STH*). *1.* Endocrinology Study Section, National Institutes of Health. BGH-1; *2.* Horner Co., Ltd., Montreal. Bovine, lot No. 17A5110X; *3.* Dr. M. Raben, human, lot No. 4.

Growth hormone was prepared immediately before use by dissolving in distilled, deionized water and adjusting to a final pH of 9.0 to 10.0 with 0.1 N NaOH.

Thyroid stimulating hormone (*TSH*). Armour Laboratories; lot IRW. Activity 1.5 to 2 U.S.P. units per mg. Estimated to contain 0.02 U.S.P. unit ACTH per mg. and negligible amounts of growth hormone. It was dissolved in distilled, deionized water just before use.

Pitressin®. Parke, Davis and Company: 20 pressor units per ml., with 0.5 per cent chlorobutanol as a preservative.

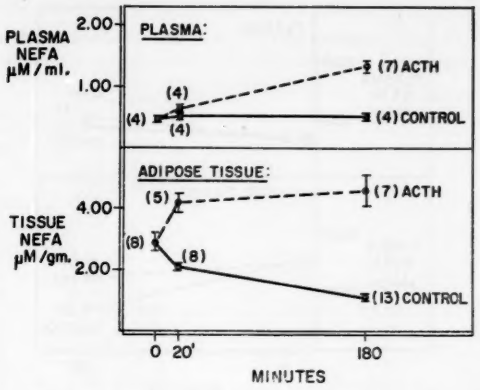

FIG. 1. INFLUENCE OF OXYCEL ACTH (10 μG. PER ML.) ON THE CONCENTRATION OF NONESTERIFIED FATTY ACIDS (NEFA) (MEAN PLUS OR MINUS S.E.) WITHIN ADIPOSE TISSUE AND RELEASED INTO A PLASMA MEDIUM DURING INCUBATION IN AIR AT 36° C.

Pitocin®. Parke, Davis and Company; 10 international units per ml., with 0.5 per cent chlorobutanol as a preservative.

RESULTS

The direct action of ACTH in promoting the release of nonesterified fatty acids from adipose tissue incubated in rat plasma is illustrated in the upper portion of Figure 1. During a three hour observation period there was no significant release of fatty acids into the medium when plasma alone, plasma and ACTH or plasma and adipose tissue were incubated. In contrast a small, but statistically significant increment in fatty acid release was already detectable in 20 minutes with a further substantial increase by 180 minutes when 10 μg. of oxycel ACTH was added to the plasma containing adipose tissue.

The lower panel of Figure 1 presents evidence that the release of fatty acids into the medium was not due either to a simple diffusion of intracellular fatty acids into the medium or to the release from the adipose tissue of an intracellular lipase which then hydrolyzed plasma triglycerides. Estimation of the fatty acid content of the adipose tissue itself revealed that fatty acids accumulated within the tissue promptly and in greater concentration than were released into the medium. Without hormonal stimulation the intracellular fatty acid content actually declined significantly over

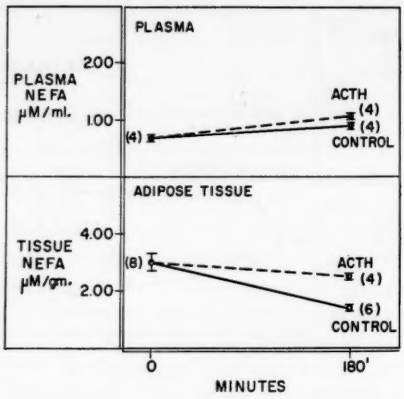

FIG. 2. INFLUENCE OF OXYCEL ACTH (10 μG. PER ML.) ON THE CONCENTRATION OF NONESTERIFIED FATTY ACIDS (MEAN PLUS OR MINUS S.E.) WITHIN ADIPOSE TISSUE AND RELEASED INTO A PLASMA MEDIUM DURING INCUBATION IN AN ATMOSPHERE OF NITROGEN AT 36° C.

time. These results suggest that ACTH stimulates the hydrolysis of neutral fat within the cell with secondary release of fatty acids into the medium. Further support for this interpretation is found in the observation that when adipose tissue is incubated in a Krebs-Ringer phosphate medium without the addition of albumin as a fatty acid acceptor, ACTH induces an increase of fatty acids only in the tissue, with none being released into the medium (10).

Figure 2 demonstrates that the lipolytic action of ACTH on adipose tissue is largely abolished by anaerobiosis. When adipose tissue is incubated in a nitrogen atmosphere there is a small loss of fatty acids from the tissue, but this is not modified by the addition of ACTH to the degree that it is aerobically. The decline in tissue fatty acids during incubation is less under anaerobic conditions and is only slightly altered by ACTH. The lipolytic action of ACTH thus appears to depend on continuing aerobic metabolism of the tissue.

Results of studies on the lipolytic potency of various preparations of ACTH and of the chemically closely related hormones, α and β melanophore stimulating hormones (α and β MSH), are found in Table II. An old sample of Armour ACTH, presumably protein in nature, and assaying 1 unit per mg., was inactive at a level of 5 μg. per ml. of plasma but active at 10 μg. and greater. This same preparation was not active in causing

TABLE II

Influence of corticotropin and melanophore stimulating hormones (MSH) on production of nonesterified fatty acids (NEFA) from adipose tissue in vitro

Hormone	No. of determinations	Dose	μM NEFA produced/100 mg. adipose tissue/3 hrs. (±S. E.)	p
		μg./ml.		
Control	28		0.19 ± 0.06 (±0.29 S. D.)	
"Protein" ACTH	5	5	−0.08 ± 0.04	*
"Protein" ACTH	7	10	0.70 ± 0.17	<0.01
"Protein" ACTH	4	50	1.55 ± 0.04	<0.01
"Protein" ACTH	4	100	1.64 ± 0.28	<0.01
Oxycel ACTH	8	1	1.08 ± 0.15	<0.01
Corticotropin "A"	2	8	2.39	†
Corticotropin "A"	1	16	2.30	†
α_1–α_2 ACTH	6	10	1.65 ± 0.09	<0.01
α_1–α_2 ACTH	3	20	1.73 ± 0.25	<0.01
γ pool ACTH	2	6	1.38	†
γ pool ACTH	1	12	2.42	†
δ–1 ACTH	1	10	1.74	†
δ–1 ACTH	1	20	1.87	†
$\beta P_s(3)$ ACTH	1	10	2.27	†
$\beta P_s(3)$ ACTH	1	20	1.56	†
C–10	3	10	0.16 ± 0.08	*
C–10	1	20	0.29	*
α–MSH	2	100	0.35	*
α–MSH	2	200	0.53	*
β–MSH	2	200	0.27	*
β–MSH	2	400	0.21	*

* Not significantly different from control.
† Exceeds control by more than two standard deviations.

TABLE III

Inactivation of ACTH with NaOH and H_2O_2, and the reactivation of H_2O_2–ACTH with cysteine

Treatment	No. of determinations	Dose	μM NEFA produced/100 mg. adipose tissue/3 hrs. (±S. E.)	P
		μg./ml.		
Control	28		0.19 ± 0.06 (±0.29 S. D.)	
α_1–α_2	6	10	1.65 ± 0.19	<0.01
α_1–α_2–H_2O_2	1	10	0.23	*
α_1–α_2–H_2O_2	1	20	0.21	*
α_1–α_2–H_2O_2–cysteine	1	10	1.24	†
α_1–α_2–H_2O_2–cysteine	1	20	1.07	†
α_1–α_2 NaOH 25°	1	10	1.58	†
α_1–α_2 NaOH 100°	2	8	0.30	*
Oxycel ACTH	8	1	1.12 ± 0.21	<0.01
Oxycel ACTH	7	10	2.11 ± 0.31	<0.01
Oxycel ACTH–H_2O_2	4	2	0.23 ± 0.06	*
Oxycel ACTH–H_2O_2	4	20	0.53 ± 0.19	*
Oxycel ACTH–H_2O_2–cysteine	4	2	2.28 ± 0.67	<0.01
Oxycel ACTH–H_2O_2–cysteine	4	20	3.44 ± 0.02	<0.01
Oxycel ACTH–NaOH 25°	4	10	1.95 ± 0.26	<0.01
Oxycel ACTH–NaOH 100°	4	10	0.05 ± 0.08	*

* Not significantly different from control.
† Exceeds control by more than two standard deviations.

ketosis in the rat (4). One μg. of oxycellulose ACTH actively stimulated fatty acid release. Corticotropin A, the pure, chemically characterized peptide ACTH consisting of 39 amino acids, was active at two doses, but limitation of supply precluded further testing. Both α_1-α_2 ACTH and the γ-pool, mixtures of ACTH peptides obtained in the purification of oxycellulose ACTH by countercurrent distribution by Shepherd and associates (11) and having high ACTH potency, had lipolytic activity. Both of these samples have potent ketogenic and adipokinetic activities in rats and mice (4, 12). δ-1, a fraction of lower ACTH potency, was lipolytic, although it had previously been found not to be ketogenic. On the other hand, it is of interest that Steelman (12) has found it to be a potent adipokinetic agent in the mouse. βP_8 (3), a peptide derived by graded pepsin digestion of β-corticotropin such that 10 amino acids were removed from the tail of molecule, leaving a 29 amino acid peptide with persisting ACTH activity, had lipolytic activity *in vitro* and adipokinetic *in vivo* (12), although ketogenic activity was much decreased (4). Finally, Lederle fraction C-10, a product of chymotrypsin digestion of β-corticotropin and comprising the peptide chain from amino acid 16 to 36 (3), was quite inert, as it has been in all parameters tested to date.

α and β MSH, 13 and 18 amino acid peptides with striking structural similarities to the corresponding N-terminal amino acid sequence of β ACTH (13), were inactive in high doses in the lipolysis test. α MSH is free of adipokinetic activity (12), and β MSH does not stimulate ketosis (4).

Previous studies have shown that various procedures which may inactivate and reactivate ACTH as judged by adrenal ascorbic depleting activity usually have comparable effects on the extra-adrenal activities (4, 5). (The effects of pepsin and chymotrypsin digestion on ACTH activity have already been described.) ACTH may be inactivated by oxidation with H_2O_2 and reactivated by incubation with a suitable reducing agent, such as cysteine (14). Table III presents data showing that the *in vitro* lipolytic activity of α_1-α_2 and oxycel ACTH on adipose tissue follows a similar pattern with H_2O_2 and cysteine treated ACTH. Activity was lost on oxidation with H_2O_2 and restored by reduction with cysteine. ACTH resists mild alkali treatment (0.1 N NaOH at 25° C. for 24 hours) but is destroyed by boiling in 0.1 N NaOH for 20 minutes (4). The lipolytic activity of α_1-α_2 and oxycel ACTH behaved in a corresponding fashion. These results lend strength to the view that the lipolytic response is a specific activity of ACTH and not due

to contamination with some other pituitary hormone.

Further support for this interpretation is found in Table IV which compares the lipolytic activity of ACTH with other pituitary hormones. Whereas 1 μg. of oxycel ACTH stimulated fatty acid release, growth hormone (STH) and thyroid stimulating hormone (TSH) showed no or at best limited activity. One sample of bovine and one of human STH were inactive even at a level of 1 mg. per ml. while another bovine STH sample was active at levels of 100 to 200 μg. per ml. The TSH preparation induced lipolysis at 500 and 1,000 μg. but not at 100 μg. Preliminary studies indicate that in contrast to ACTH, STH activity is not eliminated by oxidation with H_2O_2. The known contamination of these hormones with ACTH does not account for their lipolytic activity. It is not clear why increasing levels of the active STH and TSH preparations did not bring about as great fatty acid release as did the maximally effective dose of ACTH. The different behavior of ACTH compared to STH and TSH in this system essentially eliminates further need to consider the possibility that the ACTH effects are due to contamination with either one or both of the above hormones. Both Pitressin® and Pitocin® were likewise inactive, an important consideration in view of Ingle and Li's finding that vasopressin was responsible for the presumed extra-adrenal effect of ACTH in the muscle work test (15).

DISCUSSION

The direct action of ACTH on fatty acid production by adipose tissue represents the most clear demonstration yet that this hormone is capable of influencing metabolic processses under circumstances in which the mediation of adrenal cortical secretion is completely eliminated. The only other extra-adrenal *in vitro* effect of ACTH with which we are familiar is that reported by Randle and Young (16). These investigators found that corticotropin enhanced the action of insulin on glucose uptake when added to rat diaphragm *in vitro*. The significance of their results is

TABLE IV

Influence of pituitary hormones on production of NEFA from adipose tissue in vitro*

Hormone	No. of determinations	Dose	μM NEFA produced/100 mg. adipose tissue/3 hrs. (±S. E.)	p
Control	28		0.19 ± 0.06 (±0.29 S. D.)	
Oxycel ACTH	4	0.5 μg./ml.	0.37 ± 0.11	†
Oxycel ACTH	8	1 μg./ml.	1.08 ± 0.15	<0.01
Oxycel ACTH	4	2 μg./ml.	1.59 ± 0.11	<0.01
Oxycel ACTH	10	5 μg./ml.	2.37 ± 0.28	<0.01
Oxycel ACTH	7	10 μg./ml.	2.11 ± 0.31	<0.01
Oxycel ACTH	2	100 μg./ml.	2.16	
Oxycel ACTH	2	1,000 μg./ml.	2.16	
Human STH	2	500 μg./ml.	0.00	†
Human STH	2	1,000 μg./ml.	0.17	†
STH–R50109	4	500 μg./ml.	0.18 ± 0.08	†
STH–R50109	5	1,000 μg./ml.	0.26 ± 0.07	†
STH–17A5110x	3	10 μg./ml.	−0.05 ± 0.13	†
STH–17A5110x	5	100 μg./ml.	0.53 ± 0.14	<0.05
STH–17A5110x	6	200 μg./ml.	0.88 ± 0.12	<0.01
STH–17A5110x	10	500 μg./ml.	0.87 ± 0.10	<0.01
STH–17A5110x	6	1,000 μg./ml.	0.85 ± 0.15	<0.01
TSH–IRW	4	100 μg./ml.	0.34 ± 0.06	†
TSH–IRW	4	500 μg./ml.	0.86 ± 0.27	<0.01
TSH–IRW	4	1,000 μg./ml.	1.47 ± 0.35	<0.01
Pitressin®	3	0.02 U/ml.	0.18 ± 0.11	†
Pitressin®	3	0.20 U/ml.	0.34 ± 0.12	†
Pitressin®	3	2.00 U/ml.	0.44 ± 0.22	†
Pitocin®	3	0.01 U/ml.	0.19 ± 0.12	†
Pitocin®	3	0.10 U/ml.	0.25 ± 0.06	†
Pitocin®	3	1.00 U/ml.	0.37 ± 0.20	†

* Abbreviations used are as follows: NEFA, nonesterified fatty acid; ACTH, adrenocorticotropic hormone; STH, growth hormone; TSH, thyroid stimulating hormone.

† Not significantly different from control.

clouded, however, by the fact that they found peroxide and periodate "inactivated" corticotropin to be still active in this test, whereas so far we have found that all other extra-adrenal activities of ACTH have disappeared following peroxide treatment of the hormone.

On the basis of the data thus far available, it would appear that the action of the hormone is on the hydrolysis of neutral fat within the cell. This interpretation, however, cannot be accepted with finality until analysis is made of the tissue and medium for the glycerol moiety or until the specific fatty acids released are identified and estimated. Both of these procedures are planned for the near future. Gordon (17) has criticized Dole's method of analysis of nonesterified fatty acids on the grounds of inadequate specificity, with lactic acid singled out as an important interfering substance. We have tested our system for lactic acid by the Barker-Summerson method and found no change in response to ACTH stimulation (18).

The influence of ACTH on adipose tissue has a parallel in its action on the adrenal cortex. A loss of cholesterol esters and triglyceride fat from the adrenal cortex has long been known as an early and characteristic consequence of ACTH action (7). Surprisingly little specific information is available, however, about the fate of the lipid other than cholesterol in the adrenal. It is pertinent to the present study that lipase activity of the adrenal cortex has been reported to increase during ACTH stimulation (19). It would seem reasonable to suggest that the fatty acids from cholesterol esters and neutral fat in the adrenal cortex might serve as a source of fatty acyl-CoA and acetyl-CoA from which the steroids may be synthesized and that ACTH may serve to initiate the hydrolysis of fatty acid esters. Further support for the analogy between ACTH action on the adrenal cortex and adipose tissue is found in the fact that when the lipolytic effect on adipose tissues is studied in artificial media instead of plasma, the requirements for maximal activity appear to be very similar to those reported necessary for stimulation of adrenal slices by ACTH *in vitro* (10, 20). These same conditions are not required for the *in vitro* lipolytic action of epinephrine and norepinephrine (10, 21, 22).

It was surprising to find that growth hormone and TSH, which are active in inducing fatty liver and ketosis in the intact animal, were relatively impotent compared to ACTH in stimulating fatty acid release from adipose tissue *in vitro*. This is particularly noteworthy since Raben and Hollenberg (23) have recently reported that 0.5 to 1.0 mg. of growth hormone will bring about a sustained increase in plasma fatty acids in the diabetic dog. Approximately the same quantity of growth hormone was found by Winegrad, Shaw and Renold to be necessary to demonstrate an action on glucose uptake and lipogenesis in rat adipose tissue *in vitro* (24). The possibility exists, as previously suggested by Krahl (25), that growth hormone may be converted to a different compound in the body before it exerts some of its effects. In any case, caution is necessary in extrapolating dose requirements from *in vitro* to *in vivo* conditions.

While the minimal dose of ACTH necessary for the lipolytic effect is many-fold less than that of growth hormone and TSH, it should be emphasized that this dose is still tremendously greater than that required to stimulate the adrenal cortex *in vitro* or *in vivo*. This is what one would anticipate if one assigns to ACTH a specific role of stimulating the adrenal cortex. The extra-adrenal tissues should be expected to be much less sensitive to ACTH. Neither the present nor previous studies answer the question as to whether endogenously secreted ACTH influences extra-adrenal metabolism to a significant degree, but this possibility deserves serious consideration.

The *in vitro* lipolytic action of ACTH is not unique for this hormone, epinephrine and norepinephrine having also been shown to be highly active (21, 22). Other hormones which have been tested and found inactive in this laboratory are prolactin (500 μg. per ml.), triiodothyronine (50 μg. per ml.), glucagon (100 μg. per ml.), serotonin (20 μg. per ml.) and hydrocortisone hemisuccinate (133 μg. per ml.). The figures in parentheses are the largest doses tested.

SUMMARY

1. Incubation of corticotropin with rat adipose tissue in a rat plasma medium resulted in an increase in the concentration of nonesterified fatty acids in both the tissue and the incubating medium. This was interpreted as indicating an increased

rate of lipolysis of neutral fat within the cell. The effect was inhibited during anaerobiosis.

2. Corticotropin A and various fractions in the purification of β-corticotropin as well as a product of pepsin digestion of β-corticotropin with high adrenocorticotropic hormone (ACTH) activity all had potent lipolytic activity. When ACTH activity was destroyed by digestion with chymotrypsin, by boiling in NaOH or oxidation by hydrogen peroxide, lipolytic activity was also lost. In the latter case both ACTH and lipolytic activity could be restored by reduction with cysteine. These data are interpreted as indicating that the lipolytic activity is an intrinsic property of corticotropin.

3. Compared to ACTH, growth hormone and thyroid stimulating hormone had low lipolytic activity. One sample of bovine and one of human growth hormone were inactive at 1.0 mg. per ml. Another bovine growth hormone was active at 100 to 200 μg. per ml. and one sample of thyroid stimulating hormone was active at 500 μg. per ml. Prolactin, Pitressin®, Pitocin®, triiodothyronine, hydrocortisone and serotonin were inactive.

4. These results are discussed in relation to the other known actions of ACTH on the adrenal cortex and extra-adrenal tissues.

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QUANTITATIVE MEASUREMENT OF HEMATOPOIETIC CELLS OF THE MARROW [1]

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In recent years with the availability of more accurate methods, attention has been directed towards the quantitative aspects of both erythropoiesis and leukopoiesis. It has been difficult to measure the precursor cells in the marrow, and their exact number still remains in question. Previous estimates of total marrow cells have utilized three general methods of approach: *a*) direct measurements of volume and cellularity of marrow in animals (1); *b*) calculations based on an assumed maturation time and on the known life span of the erythrocyte (2); and *c*) calculation based on the frequency and the estimated duration of marrow mitosis (3). The first of these methods imposes formidable technical difficulties and cannot be performed in man; the other two involve assumptions not subject to validation.

The present report concerns a direct technique in which the various procedures and assumptions employed are scrutinized. The method employs the eythroid tag Fe^{59} to relate the cells in an aliquot of marrow to the total marrow, as originally proposed by Suit (4).

MATERIALS AND METHODS

1. Experimental subjects. The animals employed in these studies included healthy Sprague-Dawley rats weighing 200 to 300 Gm., New Zealand white rabbits weighing 2 to 3.5 Kg. and 2 to 3 Kg. male Rhesus monkeys. All animals had a blood hemoglobin concentration of over 13 Gm. per 100 ml.

Clinical studies were performed on male patients with controlled tuberculosis who were being subjected to thoracotomy. Prior to operation these patients were afebrile, had red counts of over 4.5 times 10^6 per cu. mm., and normal leukocyte counts.

2. Preparation and counting of marrow suspension. Rabbits and monkeys were sacrificed by intravenous injections of air or cyanide, rats by exsanguination. Immediately after death of an animal one femur was removed, and the central 60 per cent was carefully cut out, using a bone cutter for femurs of rabbit and monkey and a file and scissors for the rat femur. Marrow was either forced out of the open end of the central shaft by injection of plasma from a syringe into the opposite end, or the shaft was split open and the column of marrow freed. The marrow was then placed in a tube containing isologous heparinized plasma or a 5 per cent albumin solution. Five ml. of plasma was used for monkey marrow, 4 ml. for rabbit and 1.5 ml. for rat marrow. The tube containing the mixture was stoppered and shaken either by hand, as described by Yoffey (5), or on a mechanical shaker at an oscillation of 100 per minute for about three minutes, until cell clumps appeared to be largely dispersed. The suspension was then pressure-filtered through a nylon mesh (MBCO Disposable Filter No. 9331, Macalaster Bicknell Company), as recommended by Thomas (6). Total volume of cell suspension was measured in a graduated centrifuge tube. The suspension was drawn into red cell counting pipettes and diluted with plasma. Counts of approximately 1,000 cells were made in a counting chamber.

An aliquot of the suspension was then centrifuged at 290 G for five minutes and the cell-free supernatant was removed. The concentrated cell suspension, after thorough mixing, was used for the preparation of smears. Differential counts of at least 2,000 marrow cells were made from Wright and Giemsa-stained smears. Unidentifiable cells in these preparations represented 3 to 6 per cent of cells counted. Reticulocyte preparations were also made by placing one drop of the thick cell suspension in a well slide on which one drop of 6 per cent new alcoholic methylene blue had been previously dried. This was mixed and allowed to stand for three minutes, after which smears were made; 2,000 non-nucleated red cells were surveyed for reticulum. All glass surfaces coming into contact with the marrow during preparation and handling of these animal and human suspensions were siliconized.

In human studies, a portion of a rib removed at operation was the source of marrow. This rib fragment was immediately wrapped in parafilm and kept on ice for a period of 30 minutes until the cell suspension could be prepared. Marrow was expressed from the rib by pressure and transferred by a capillary pipette into 2 ml. of

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plasma. Further handling and counting was performed as described for animal studies.

Details of individual experiments done to determine cell losses in preparation of marrow suspension for counting are found under Results. Radioiron techniques, including the simultaneous use of Fe^{55} and Fe^{59}, have been described previously (7). The method for the determination of hemoglobin was that of Flink and Watson (8). In studies employing paper electrophoresis, material tested was buffered at pH 6.5, 7.0 and 8.6, and the loaded strips were run at 240 volts.

3. The use of radioiron as a marrow tag. This method is based on the observation that tracer amounts of radioiron injected intravenously rapidly localize in the erythroid cells of marrow (9). The details of the methods used in animals and man to determine the ratio between the marrow aliquot and the total marrow differed somewhat, although in both instances radioiron was employed to tag the erythroid cells of the marrow.

In animal studies, radioiron was bound to freshly-drawn plasma in a concentration of less than 1 μg. iron per ml. of plasma and was injected intravenously. Rats received 1 μc. Fe^{59}, rabbits 5 μc. and monkeys 10 to 15 μc. A weighed aliquot of the solution prepared for injection was set aside as a standard. The exact amount of radioactivity injected was also determined. The animals were then killed at the time when radioactivity in circulating blood was minimal, *i.e.*, when most of the radioactivity had been cleared from the plasma and but little activity had yet appeared in circulating erythrocytes. This was found to be at 5 hours in the rat, 6 hours in the rabbit and 12 hours in the monkey. At the time of sacrifice a sample of blood was drawn for radioactivity and cell counting. One femur was removed, and a cell suspension was prepared from the central 60 per cent of the shaft. Residual radioactivity in the filter and bony shells was determined. The supernatant fraction and packed cells of the final marrow suspension were counted separately.

The carcass of the animal was eviscerated and autoclaved for two hours. Bones were then easily separated from soft tissue. The radioactivity of skeleton, liver, spleen and nonskeletal carcass, as well as that of the standard aliquot solution, was determined. A shielded two-inch scintillation crystal detector at a distance of from 6 to 24 inches from the various aliquots was employed for the measurements. The central 60 per cent of the opposite femur was removed and digested in nitric acid. The remaining skeleton, including the ends of the two femurs, was similarly digested. The activity of these two fractions, plus that of a standard, was determined in a vial scintillation counter.

In studies of man, one-half μc. Fe^{59} per Kg. body weight bound to normal plasma, or in the form of the citrate salt (10), was injected intravenously 14 to 18 hours before surgical removal of a rib. Baseline studies of the patient's blood included red cell count, hemoglobin, hematocrit, recticulocyte count, total and differential leukocyte counts. Blood was drawn for radioactive determination before injection of Fe^{59}, five minutes following injection, at the time of removal of the rib, and two weeks or more after the operation. A marrow cell suspension was prepared from the rib as described previously. The radioactivity in the centrifuged cells of 1 ml. of marrow suspension was determined.

4. Formulae employed in animals.

$$\text{Cells of suspension} = \text{ml. of suspension} \times \text{cells per ml.} \qquad \text{I}$$

$$\text{Radioactivity of central femur} = \text{activity of (suspension cells + suspension supernate + filter + central bone)} \qquad \text{II}$$

$$\text{Cells of central femur} = \frac{(\text{I}) \times (\text{II})}{\text{activity of suspension cells}} \qquad \text{III}$$

$$\text{Total marrow cells} = \frac{\text{total skeleton radioactivity} \times (\text{III})}{(\text{II})} \qquad \text{IV}$$

Formulae employed in man.

$$\text{Total marrow cells} = \frac{(\text{I}) \times 0.66 \times \text{activity injected}}{\text{activity of suspension cells}} \qquad \text{V}$$

The factor 0.66 is employed in man to correct for the amount of radioactivity localized in the marrow at the time of study (see discussion of marrow localization of radioiron).

5. Comparison of Fe^{59} with other marrow tags in determination of total marrow. In animals, two other methods were employed to establish the ratio between the marrow aliquot from the central femur and marrow from the total skeleton. These involved the quantitative analysis of marrow fractions for porphyrin and hemoglobin. It was necessary to process the carcass at 5° C. in order to prevent postmortem change in these pigments. Bones exclusive of femurs were separated from the carcass and ground in a No. 5 Enterprise food grinder. The ground bones were further pulverized in a Waring Blendor® in an ethyl acetate-acetic mixture (four parts anhydrous ethyl acetate and one part glacial acetic acid). The material was transferred to a sintered glass filter, and repeatedly ground and washed with ethyl acetate-acetic mixture. The final residue was treated with boiling glacial acetic acid. Additional ethyl acetate was added to the filtrate to reconstitute the original 1:4 ratio between glacial acetic acid and ethyl acetate. The extract was then washed three times with 3 per cent sodium acetate, and porphyrins were extracted repeatedly with 10 ml. portions of 3 N HCl until extraction was complete. Porphyrin analysis was performed by using a modified Farrand fluorometer and a Corning glass filter (primary filter No. 5113 and secondary filter No. 2412). The experimental values obtained represented total fluorescence due to protoporphyrin, coproporphyrin and any other fluorescent porphyrin. The femur was similarly prepared and its porphyrin content analyzed. As a check on reproducibility of the method used, as well as on the similarity of the opposite femur, analyses were performed on the femora of three rabbits. The duplicates agreed within a range of 8 per cent.

In using hemoglobin for determination of the femur: total marrow ratio, it was recognized that a considerable portion of hemoglobin was derived from circulating erythrocytes and that this fraction might not bear a constant relationship to marrow. Therefore, radioiron-tagged erythrocytes (Fe^{55}) were injected intravenously into each animal 10 to 20 minutes before sacrifice. The specific activity of circulating hemoglobin was determined, and an appropriate correction for peripheral blood contamination was made in each marrow hemoglobin fraction according to its radioactive content. Hemoglobin extraction from marrow was performed on the ethyl acetate-acetic acid mixture described above, following porphyrin extraction, and after the mixture had been washed with 3 per cent sodium acetate. The extraction was carried out with small volumes of concentrated NH_4OH until the organic phase was colorless. The pigment was then determined by the pyridine-hemochromogen method. In recovery studies on marrow homogenates, an average recovery of 95 (plus or minus 3) per cent of added hemoglobin was achieved in four experiments. The addition of radioactive hemoglobin also indicated that no significant amounts of the radioiron were lost in the various procedures leading to extraction of hemoglobin. Ratios were established between the femur and total skeleton for both porphyrin and hemoglobin. These were compared to results obtained by the use of Fe^{59} labeling of marrow.

RESULTS

1. Studies relating to preparation of the cell suspension

There were several problems apparent in the preparation and counting of the cell suspension. It was found that removal of cells from the ends of the femurs was technically difficult due to bone spicules. Therefore, only the central 60 per cent of the femurs was employed. Even in this central portion there was an appreciable amount of marrow and radioactivity which could not be removed without excessive cell trauma. In rabbits this residue amounted to an average of about 10 per cent of the total radioactivity in the marrow. A further loss of about 20 per cent was found on the nylon filter. A significant amount of radioiron in excess of that which could be ascribed to circulating blood was also found in the supernatant fraction of the centrifuged marrow suspension. In rats this averaged 29 per cent, in rabbits 15 per cent, in monkeys 28 per cent and in man 29 per cent. These several losses of radioactivity were collectively large and their manner of production, therefore, of importance in the calculation of results. In respect to both bone and filter radioactivity, it seemed likely that this activity reflected residual marrow tissue. Activity in the bony shells could be removed by scraping the inner surfaces of the bones. Activity on the filter was, in general, proportionate to the mass of cells retained, and was similarly assumed to reflect activity from trapped cells.

The nature of the radioactivity in the marrow supernatant fraction seemed less certain. After a small correction for that activity attributable to circulating blood content of marrow suspension, the activity of the supernatant fluid was presumed due either to destroyed cells or to iron released from marrow cells. To test the latter possibility, tagged marrow was incubated for two hours at room temperature in saline, in iron-deficient plasma, and in saline solutions containing 0.4 to 4 per cent disodium Versenate®. The supernatant fraction in saline and in the iron-deficient plasma contained 12 per cent of the total activity, similar to the amount in the original marrow supernatant fraction at zero time. Samples in versene contained 14 and 15 per cent. It was concluded that no appreciable amount of iron could reflux from the cell even in the presence of unsaturated transferrin or other chelates.

An attempt was then made to obtain positive evidence that the radioiron was derived from broken cells. A tagged marrow suspension of high activity was obtained by employing doses of 200 μc. to 500 μc. of Fe^{59} in donor rabbits. The supernatant fraction from this marrow suspension was concentrated by lyophilization. The centrifuged cells of the suspension were broken by repeated freezing and thawing. The two fractions, containing similar concentrations of radioactivity, were subjected to paper electrophoresis of pH 7.0, 6.5 and 8.6. Both showed a similar distribution of radioactivity among protein fractions (Figure 1). In the supernatant fraction, 80 per cent of the activity moved in the hemoglobin band, while 74 per cent of the activity of the broken cells was in the hemoglobin fraction. It was concluded that the form of radioiron in the supernate was consistent with derivation from broken cells and that most of the radioactivity was present as hemoglobin. Centrifuging the supernatant fraction at 2,000 G for one hour did not result in any sepa-

TABLE I

Distribution of various nucleated cell types of human marrow *

	Direct smear	In marrow suspension
Granulocytic forms	56.0	56.4
Segmented	12.2	12.4
Band	20.4	20.7
Metamyelocyte	12.9	11.7
Myelocyte	10.5	11.5
Lymphocytic and miscellaneous	4.6	4.1
Eosinophils and basophils	2.4	2.7
Blasts	0.1	0.0
Nucleated red blood cell	35.0	31.7
Smudges	1.9	5.1

* Counts listed are an average of five marrow preparations with counts of 4,000 nucleated red cells on each. Figures are expressed on a percentage basis.

ration of radioactivity, so it was unlikely that the activity was contained in cytoplasmic particles of any appreciable size.

It is our impression that losses due to cell breakdown in the suspension are unavoidable with the present technique. Variations in the filtration process or in the frequency and force of shaking had surprisingly little effect on the amount of radioactivity in the supernatant fraction. Centrifugal forces up to 1,800 G applied to the cell suspension for periods of 15 minutes did not result in an increased radioactivity in the supernatant fluid after mixing and recentrifugation of the marrow cells.

The next consideration was whether or not destruction of marrow cells and loss through filtration altered the relative incidence of the various cell types in the suspension counted. Smears were made of cells remaining on the nylon filter, and differential counts were compared with those of the final cell suspension. While no significant difference was observed, except for disappearance of megakaryocytes from the suspension, the differential counts made from the filter were not considered satisfactory for accurate counting.

Studies were then carried out on five ribs obtained from patients at operation. The nucleated cell differential (4,000 nucleated cells counted) on direct smear was compared with that of the cell suspensions. The values obtained (*cf.* Table I) are interpreted to indicate no recognizable alteration in frequency of nucleated cell types.

Since certain calculations assume that all mature erythrocytes in the marrow suspension represent circulating blood, it was considered desirable, if possible, to verify this assumption. Mature erythrocytes tagged with Fe^{59} were injected into rabbits four to six minutes before sacrifice. To provide extreme conditions, one group of rabbits was made anemic by repeated bleedings and a second group was made polycythemic by transfusion several days before injection of tagged cells. The specific activity of adult red cells in circulating blood and in marrow suspensions for these two groups of animals was determined. The ratio of marrow specific activity to blood activity in six rabbits rendered polycythemic by transfusion was 1.03 plus or minus 0.18 and 1.19 plus or minus 0.12 in four rabbits made anemic by bleeding. These data gave no indication of sequestration of erythrocytes in the marrow, for such cells unmixed with recently injected red cells would result in a specific activity less than unity.

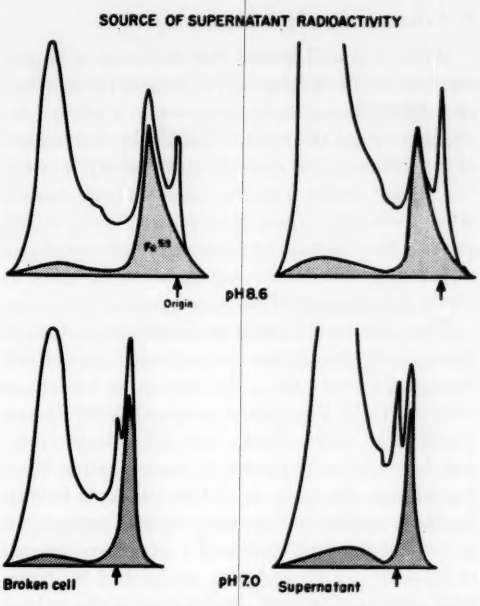

FIG. 1. PROFILES OBTAINED BY PAPER ELECTROPHORESIS OF A CONCENTRATED SOLUTION OF THE SUPERNATE OF A MARROW CELL SUSPENSION (RIGHT) AND THE LYSED MARROW CELLS (LEFT)

The clear area is the densitometry reading of the stained protein, the cross-hatched area indicating the distribution of radioactivity in the same strip. It will be observed that a similar relation is found between radioactivity and protein profiles in the two preparations run at two different pH concentrations.

2. Determination of total marrow

While it is well known that radioiron is largely localized in the developing red cells of the marrow, and should thus act as a marrow tag, it seemed desirable to check the results obtained by this method of determining the ratio of radioactivity between the central femur and the total skeleton against other methods. Since the erythroid cells of the marrow have high concentrations of both porphyrin and hemoglobin, these substances were used as chemical indicators of marrow mass.

The ratio of the femur radioactivity to that of the total skeleton in four normal rabbits was 0.048 (range, 0.04 to 0.06). In four other rabbits of similar weight, the ratio of marrow porphyrin was found to be 0.052 (0.043 to 0.055); in the marrow hemoglobin, exclusive of contaminating blood hemoglobin, the ratio was 0.040 (0.035 to 0.045). In these studies on normal rabbits, neither the spleen nor the liver contained a significant amount of marrow. This fact was established by histologic examination, and, in the case of the spleen, by finding less than 0.05 per cent of the injected radioiron in the spleen as compared to 59 per cent in the skeletal marrow.

Skeletal localization of radioiron is an important consideration when the radioiron method is extended to man. In animals studied, the skeletal localization of radioiron at the time of minimal blood activity was determined by direct carcass analysis. Since it was impossible to make similar studies in man, data from the three animal species were used to derive the per cent localization in man. Distribution of radioactivity between skeleton and other body tissues and in circulating blood at the time of sacrifice is shown in Table II. Since there were varying amounts of activity not yet deposited in body tissues, allowance was made for this on the basis of distribution already present in the tissues. These corrected figures then indicated a marrow localization of 70 per cent in the rat, 69 per cent in the rabbit and 66 per cent in the monkey. It was assumed on this basis that two-thirds of the activity in man would be similarly localized in the erythroid marrow. This refers only to the tissue-marrow distribution at the time of minimal blood activity and only in the normal steady state. It is known that in all of these species, including man, only about 10 per cent of the injected radioiron remains in tissues at two to three weeks after injection. In four patients followed subsequent to the determination of marrow cellularity, 88 per cent of the injected radioactivity was estimated to be present in circulation at three to five weeks.

3. Cellularity of the marrow in animals and man

The data in rats, rabbits, monkeys and man obtained by the radioiron method described are summarized in Table III. The results are expressed in cells per Kg. of body weight to permit comparison between species.

DISCUSSION

The measurement of marrow cellularity requires the preparation and accurate counting of a marrow aliquot and the relation of this marrow aliquot to total marrow. This report is concerned largely with the problems presented by such a method which has previously been briefly described by Suit (4).

It is assumed that marrow is reasonably homogenous throughout with respect to its composition of hematopoietic cells. While Kindred (1)

TABLE II

Carcass localization of radioiron

Species	Number of subjects	Time after radioiron injection	Skeleton	Tissues	Blood	Calculated total skeletal activity*
		hrs.	%	%	%	%
Rat	4	5	56 (49–67)	24	20 (17–22)	70
Rabbit	4	6	59 (56–65)	26	15 (2–18)	69
Monkey	4	12	64 (53–84)	32	4 (2–8)	66
Man	8	15			2 (1–6)	

* This figure arrived at by assuming a distribution of blood activity between skeleton of other body tissues similar to that of the isotope already localized extramuscularly.

has suggested a 10 per cent difference in the relative number of nucleated erythroid cells in the long bones versus central marrow, a difference of this magnitude is not considered to be critical in the present study. Differential counts of hematopoietic cells performed on human marrow from two or more areas have no important variations. It has been our experience that a marrow aliquot of some size is required for determination of marrow cellularity as outlined here. In these studies on animals and man, in excess of 200×10^6 nucleated cells were contained in the cell suspension. Studies with marrow aspirates containing fewer nucleated cells and more peripheral blood contamination have as yet been unreliable in our laboratory. Since Suit (4) has obtained figures similar to our own employing considerably smaller amounts of radioactivity and obtaining human marrow by needle aspiration, it seems likely that this method may be adaptable to smaller marrow samples.

Surprising numbers of marrow cells, approximately 50 per cent, were left in the bony shell or filter, or were broken down in the cell suspension. The recovery of marrow cells probably could have been improved by more complete filtration of the cells through the nylon filter. Variations in the specific procedure used in preparation of the suspension, *i.e.,* filtration and shaking, seemed to have little effect on the degree of cell breakdown. There was no evidence from differential counts on smears made directly from the marrow, from the cells remaining on the nylon filter, or from the marrow suspension, that there had been any measurable change in the ratio of nucleated hematopoietic elements through the preparation of the suspension. Not only the erythroid-myeloid ratio, but also the myelocytic differential count remained essentially unchanged. Evidence has been presented elsewhere (11), however, that the destruction of nucleated cells is appreciably greater than that of non-nucleated erythrocytes in the suspension.

The contamination of the marrow suspension with leukocytes could be determined from the peripheral leukocyte count and the number of mature erythrocytes in the suspension. By these calculations the circulating granulocyte contribution from the blood was less than 0.1 per cent of the myelocytic cells in the marrow preparation. Likewise, the contribution of radioactivity from the circulating blood in marrow suspension present was less than 1 per cent in all species studied and could therefore be ignored in calculations.

TABLE III

Marrow cellularity

Species	Number of subjects	Nucleated marrow cells/Kg. $\times 10^9$	
		Average	Range
Rat	4	17	13–22
Rabbit	5	12	9–18
Monkey	5	34	22–44
Man	7	18	11–30

One can accept the radioiron values obtained from marrow aliquot and total skeleton in animals with some assurance, since they represent direct measurements and agree with two other marrow tags (hemoglobin and porphyrin). The use of this procedure in man, however, involves an additional assumption, *i.e.,* that a given amount of iron has localized in the marrow. A figure of two-thirds was arrived at by comparison of data in three animal species. The agreement of the figure obtained in monkeys with the figures obtained in other animals is of particular significance, since activity in circulation at the time of marrow sampling is quite similar to that in man.

The data obtained in these four species are meaningful when they are broken down into figures for erythrocytic and granulocytic cell series. This will be done in detail in a following article (12). It is of interest, however, to compare the over-all figures presented here, varying from 12 to 34×10^9 nucleated marrow cells per Kg. body weight with results obtained by different methods. Kindred (1) found 25×10^9 cells per Kg. in rats, and Patt (3) estimated 49, 17 and 12×10^9 per Kg. for the combined nucleated erythroid and myeloid cells of the marrow of rats, dogs and man, respectively. Osgood (2) estimated 34×10^9 erythroid and myeloid cells per Kg. in man, and Suit (4), employing the technique used here, found 16×10^9 marrow cells per Kg. body weight in man. These various data therefore are in general agreement concerning the size of the total hematopoietic cellular mass.

SUMMARY

A method has been described for determination of the total cellularity of hematopoietic tissue of the marrow, exclusive of megakaryocytes. This has been accomplished by determining the number of cells in an aliquot of marrow and by relating this aliquot to the total marrow. Observations concerning possible technical errors in the method and results found in three species of animals and in man have been described.

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QUANTITATIVE MEASUREMENT OF THE ERYTHROCYTIC AND GRANULOCYTIC CELLS OF THE MARROW AND BLOOD [1]

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In the preceding article, a method for measuring total marrow cellularity was described (1). The purpose of this report is to relate numerically (*a*) the erythroid marrow to the circulating red cell mass, and (*b*) the granulocytic marrow to the circulating granulocytic leukocytes. Results in three animal species and in man are reported, and are compared with data obtained by other methods.

MATERIALS AND METHODS

Studies were performed on Sprague-Dawley rats weighing between 200 to 300 Gm., New Zealand white rabbits weighing 2 to 3½ Kg., and 2 to 3 Kg. male Rhesus monkeys. All animals were given approximately 15 mg. per Kg. of iron as iron dextran [2] at least two weeks before the study to insure adequate iron stores. Only those animals with hemoglobin levels above 13 Gm. were used. Reticulocyte counts in rabbits were required to be below 4 per cent and those of monkeys and rats below 3 per cent before the animals were considered suitable for study.

Clinical studies were performed on male patients with controlled tuberculosis who were being subjected to thoracotomy. Prior to operation, these patients had normal leukocyte counts and were afebrile, but plasma iron values were usually subnormal. On the morning of operation their red counts were above 4.5×10^6; their reticulocyte counts were less than 2 per cent. They had not been transfused during the previous six months.

I. *Erythrocytic series*

A. Studies of the circulating erythrocytes. A red count was performed by enumerating a minimum of 1,000 cells from two or more pipettes in two counting chambers. Reticulocyte counts were performed on dried smears of blood stained with new methylene blue. One thousand cells were surveyed. Blood volume measurements were performed by the Cr^{51} or P^{32} techniques on only a few of the animals studied. In the remainder of the animal experiments and in clinical studies with patients, volumes were calculated from Kg. body weight according to normal values previously established in our laboratory. The value of 60 ml. per Kg. was used for rats, rabbits, monkeys and for the patients studied. While the volume for normal ambulatory man was previously found to be about 68 ml. per Kg. (2), additional studies indicated that the usual value for patients at bed rest was approximately 60 ml. per Kg.

B. Studies of the noncirculating erythropoietic tissue. In animals, a cell suspension of marrow was prepared from the central 60 per cent of the femur of the sacrificed animal. In human studies, a portion of rib removed at operation was the source of marrow. Dispersion of marrow cells was achieved by shaking and by filtering the suspension through a nylon mesh. Total cell counts, employing red cell pipettes and counting chambers, were performed with the enumeration of approximately 1,000 cells. The suspension was then concentrated by centrifugation. Smears were made directly from this concentrate, stained with Wright and Giemsa, and a 2,000 cell differential count was performed. In this study eosinophil and basophil forms were excluded from the granulocytic series. Additional smears were made of the marrow cells stained with new methylene blue, and 2,000 non-nucleated red cells were examined for reticulum. Details of this method are presented in the preceding article (1).

The quantitative relationship between the aliquot of marrow in the central shaft of the animal femurs or from the rib in man and the total marrow was established through the use of Fe^{59} as a marrow tag. Iron was injected either bound to the β_1 globulin of fresh plasma or in a manner to insure binding *in vivo* (3). In animals, 2 to 5 μc. per Kg. of Fe^{59} and in man ½ μg. per Kg. were employed. Marrow was obtained at the time of minimal circulating radioactivity for preparation of a cell suspension, *i.e.*, 5 hours in the rat, 6 hours in the rabbit, 12 hours in the monkey and 15 hours in man.

In animal studies the radioactivity, cell count and composition of the marrow suspension were determined. Losses in preparation of the suspension, as reflected in

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the residual radioactivity on bone fragments, filter, and in the supernate of the marrow suspension, were also determined as an index of the suitability of the preparation. In animals the total skeleton was isolated from the autoclaved carcass and its radioactive content determined.

From the specific activity of cells in the marrow suspension and the total marrow activity of the skeleton, it was possible to calculate the number of marrow hematopoietic cells. In man, it was necessary to assume that a certain percentage of the injected radioiron would be located in the marrow at the time of marrow sampling. On the basis of animal studies (1), this figure is taken to be 66 per cent.

C. Formulae employed—Calculations employed

1. *Circulating erythron. Total circulating erythrocytes* are calculated from the product of the red count per ml. and the total blood volume in ml. *Circulating reticulocytes* are represented by the product of the total circulating erythrocytes and the per cent of reticulocytes. *Circulating mature erythrocytes* equal the difference between total circulating erythrocytes and circulating reticulocytes.

2. *Noncirculating erythron.* In animals, total marrow cellularity (Ctm) is determined from the cells per ml. marrow suspension (Cs), the radioactivity of the centrifuged cells in 1 ml. marrow suspension (Rs), and total marrow radioactivity (Rtm) as follows:

$$\text{Ctm} = \frac{\text{Rtm} \times \text{Cs}}{\text{Rs}}. \qquad \text{(I)}$$

It should be noted that total marrow radioactivity includes, along with the activity of the isolated skeleton, the radioactivity of the marrow aliquot and the activity lost in its preparation (*i.e.*, on bones, in filter and in supernate).

Essentially the same formula is employed for man except that total marrow radioactivity may not be determined directly. In its place the injected dose (Ri) times a factor of 0.66 is substituted to allow for the per cent localized in the skeleton:

$$\text{Ctm} = \frac{\text{Ri} \times 0.66 \times \text{Cs}}{\text{Rs}}. \qquad \text{(II)}$$

In these studies the radioactivity in marrow attributable to the circulating blood was ignored, since such activity represented less than 2 per cent of the counts in the marrow. In the event of greater peripheral blood contamination this would be important, and appropriate corrections would be required in the calculation of results.

The percentages of nucleated red cells, reticulocytes, and adult erythrocytes were obtained from the differential cell counts in the marrow suspension. Knowing the total number of marrow cells, we could then calculate the absolute number of each of these. Other results (1) indicate that there is no appreciable number of mature nonreticulated erythrocytes in the fixed marrow, so the number of these cells was taken to reflect the amount of circulating blood mixed with marrow. From this figure and from the per cent of reticulocytes in circulation, a correction in the total marrow reticulocyte count was made as follows:

$$\text{True marrow reticulocytes} = \text{Rm} - \left(\text{Em} \times \frac{\text{Rb}}{100 - \text{Rb}}\right). \qquad \text{(III)}$$

When Rm is total marrow reticulocyte, Em is erythrocytes without reticulum in marrow suspension, and Rb is the per cent reticulocytes in the circulating blood. The erythron has thus been arbitrarily divided into four categories of cells: (*1*) nucleated red cells of the marrow, (*2*) reticulocytes of the marrow, (*3*) reticulocytes in circulation and (*4*) mature erythrocytes in circulation.

II. *Granulocytic series*

Total granulocytes in circulation were determined from the concentration of granulocytes and the estimated blood volume, as described for circulating red cell estimates. A total of 500 leukocytes was enumerated in the counting chamber in determining the leukocyte count per cu. mm. of blood, and a differential count of 200 white cells was performed on the Wright-stained peripheral blood smear. Differential cell counts on smears of the marrow suspensions were performed on 2,000 to 4,000 nucleated cells. *Total marrow granulocytic forms* were determined from the total marrow cellularity (Formulae I and II) times per cent granulocytic cells. Morphologic criteria employed arbitrarily to separate the granulocytic series into designated categories were those generally employed (4). From the differential counts of the granulocytic series, the numbers of myelocytes, metamyelocytes, bands and segmented forms were determined.

RESULTS

I. Erythron

Data for the number of erythroid forms in the marrow and blood of five rats, five rabbits and five monkeys are summarized in Table I, and comparable data in nine human subjects are shown in Table II. The number of nucleated red cells is comparable in three of the four species when expressed as cells per Kg. body weight.[3] Thus, in the rat there were 5.5×10^9 nucleated erythrocytes per Kg., in the rabbit there were 5.8, and in man, 5.4, while in the monkey a value of 9.2 was obtained. In these same four species the combined reticulocytes of marrow and blood were 14.5, 16.4,

[3] In all species the red cell hemoglobin concentration was similar, individual values varying between 31 and 34 per cent. Red cell volume, however, showed species' differences. In the rat, mean corpuscular volume averaged 55.5 cu. mm.; in the rabbit the volume was 67; in the monkey, 68; and in man, 86 cu. mm.

TABLE I

Animal erythroid cells

Species	Number of animals	Nucleated red blood cells/ Kg. body weight $\times 10^9$	Marrow reticulocytes/ Kg. body weight $\times 10^9$	Circulating reticulocytes/ Kg. body weight $\times 10^9$	Mature red blood cells/ Kg. body weight $\times 10^9$
Rat	5	5.55 ± 0.59*	6.05 ± 1.41	8.46 ± 3.55	493 ± 62
Rabbit	5	5.8 ± 2.3	4.8 ± 1.4	11.6 ± 2.6	370 ± 28
Monkey	5	9.2 ± 1.9	6.1 ± 1.6	4.9 ± 3.1	380 ± 15

* Standard deviation.

8.95 and 11.0 × 10^9 cells per Kg., respectively. Of this total compartment, the percentage in the marrow [4] was 42, 29, 65 and 56 per cent. Average figures for red cells in these four species were 493 × 10^9 Kg. in rats, 370 in rabbits, 309 in man and 380 in monkeys.

II. Granulocytic series

Summary data of measurements of the granulocyte series of marrow and blood are shown in Tables III and IV. Circulating granulocytes in rats, rabbits, monkey and man averaged 0.17, 0.24, 0.44 and 0.30 × 10^9 Kg. Total marrow granulocyte forms of these species were 6.7, 4.8, 15.4 and 11.4 × 10^9. The accompanying tables summarize further distribution of relative numbers of cells at various stages of their development.

[4] Other studies have indicated that a 20 per cent differential destruction of nucleated, as compared to non-nucleated, erythrocytes may occur in preparation of the marrow cell suspension (5). Thus, the actual value of marrow reticulocytes may be less than this recorded value by 0 to 20 per cent. Assuming the maximum difference, this would reduce the estimates summarized in Table I to 4.8, 3.8, 4.9 and 4.6 × 10^9 marrow reticulocytes per Kg.

DISCUSSION

Various experimental approaches have been employed to estimate the number of marrow precursors of the circulating blood elements. It is of interest to review these methods and the results obtained by their use (Table V). Kindred (6) carried out meticulous and detailed studies in the rat in which he counted both the number of cells in histologic sections and the volume of the marrow cavities. From these data he directly calculated total nucleated erythrocytes. Hudson and Yoffey used an experimentally determined marrow volume (7) and counted the cells in a given volume of femoral marrow (8). Osgood (9) used the known turnover rate of the mature erythrocyte and the estimated maturation time of immature erythrocytes derived from the time of reticulocyte response following alterations of marrow function

TABLE II

Human erythroid cells

Subject	Nucleated red cells (total cells/ Kg. × 10^9)	Marrow reticulocytes (total cells/ Kg. × 10^9)	Circulating reticulocytes (Cells/cu. mm. × 10^6)	Circulating reticulocytes (Total cells/ Kg. × 10^9)	Circulating mature erythrocytes (Cells/cu. mm. × 10^6)	Circulating mature erythrocytes (Total cells/ Kg. × 10^9)
T. S.	8.51	10.92	0.042	2.50	4.69	281
K. A.	2.28	2.88	0.073	4.44	5.22	313
B. T.	3.75	7.03	0.064	3.83	5.32	319
R. B.	4.46	3.34	0.041	2.62	5.14	308
C. Ba.	5.63	5.11	0.035	2.13	5.91	354
P. M.	4.76	4.37	0.071	4.09	5.46	328
B. N.	6.48	4.10	0.066	4.00	5.11	307
C. Bo.	5.24	6.23	0.029	2.04	4.87	292
C. O.	7.15	7.63	0.056	3.35	4.66	279
Average	5.36	5.73	0.053	3.22	5.28	309
Geometric mean	5.04	5.25	0.052	3.10	5.14	308

TABLE III

Animal granulocytes per Kg. body weight

Species	Number of animals	Circulating granulocytes ($\times 10^9$)	Total marrow granulocyte forms ($\times 10^9$)	Segmented granulocytes ($\times 10^9$)	Marrow band ($\times 10^9$)	Metamyelocytes and myelocytes ($\times 10^9$)
Rat	4	0.17 ± 0.13	6.76 ± 1.76	0.45 ± 0.08	3.59 ± 0.91	2.72 ± 0.97
Rabbit	4	0.24 ± 0.06	4.84 ± 1.25	0.29 ± 0.15	2.28 ± 0.74	2.27 ± 0.87
Monkey	4	0.44 ± 0.06	15.40 ± 3.65	1.57 ± 0.80	7.11 ± 2.43	6.73 ± 1.09

in man. Patt (10) also used the known erythrocyte production rate and erythroid/myeloid ratio, but employed Japa's data (11) on frequency of mitosis in erythroid and granulocytic series, and data on duration of mitosis from other sources (12, 13). Suit (14) employed the radioiron method for his determinations. In surveying the results by these various approaches carried out in six different species, the amount of total hematopoietic tissue has been reported to range from 12 to 49 $\times$ 10^9 marrow cells per Kg. in all species.

To consider more specifically the red cell series, nucleated red cells have been reported to range from 3 to 22 $\times$ 10^9 cells per Kg. The present results fit within this range, values in rat, rabbit and man being between 5 and 6 $\times$ 10^9 per Kg. The only fraction of erythroid cells capable of cell division is within their nucleated cell population. Since the number of cells produced may be calculated from the number and life span of circulating erythrocytes, the doubling time of the nucleated red cell fraction may be estimated. In this calculation the red cell turnover is assumed to be 1 per cent per day in man and monkey (15) and 2 per cent per day in rat and rabbit (16). Figures for doubling time of nucleated red cells would be 0.56 day in rats, 0.76 in rabbits, 2.43 in monkeys and 1.7 days in man. It is recognized that these calculations are approximate, and the assumption is made that there is no great discrepancy between total erythropoietic activity by the marrow and circulating red cell turnover, *i.e.*, between total and effective erythropoiesis (17).

Seip was the first to attempt to express quantitatively the relation of marrow reticulocytes to nucleated cells of the marrow (18). He obtained a ratio of 1.7:1. In a similar study by us (5), the ratio of marrow reticulocytes to nucleated cells in man was found to be approximately 1:1. In the present study of four species the number of noncirculating reticulocytes was approximately equal to that in circulation. The rabbit showed the greatest disparity, having only 29 per cent of reticulocytes in the marrow. This could ex-

TABLE IV

Human granulocytic cells

Subject	Total marrow granulocytic forms (cells/Kg. $\times 10^9$)	Marrow myelocytes (cells/Kg. $\times 10^9$)	Marrow metamyelocytes (cells/Kg. $\times 10^9$)	Marrow band forms (cells/Kg. $\times 10^9$)	Marrow adult granulocytes (cells/Kg. $\times 10^9$)	Circulating mature granulocytes (cells/Kg. $\times 10^9$)
T. S.	17.3	2.8	3.8	6.0	4.7	0.31
K. A.	7.2	1.5	1.4	2.4	1.9	0.28
B. T.	9.5	2.4	1.5	3.5	2.2	0.19
R. B.	8.1	1.8	1.6	3.7	1.0	0.38
C. Ba.	10.3	3.8	1.7	3.1	1.7	0.28
P. M.	5.7	1.8	1.7	1.6	0.6	0.27
B. N.	10.3	2.5	1.8	4.8	1.2	0.37
C. Bo.	17.3	2.9	3.3	5.9	5.2	0.5
R. J.	21.6	4.9	8.1	3.6	4.9	0.35
G. R.	6.8	1.2	2.1	1.7	1.7	0.09
Average	11.4	2.6	2.7	3.6	2.5	0.30
Geometric mean	12.6	2.4	2.3	3.3	1.6	0.28

plain the higher circulating reticulocyte of the rabbit as compared to the rat, despite their similar red cell life span (16).

Measurements of the granulocyte series are essentially a by-product of those employed to estimate red cell precursors, since these may be related through the erythroid/myeloid ratio of the marrow. The number of marrow granulocytic forms in six species has been estimated or determined by others to be variously 4.8 to 27 $\times 10^9$ cells per Kg. In these species the circulating granulocytes range from 0.15 to 0.45 $\times 10^9$ cells per Kg. (4). Our own studies in four species indicate a range of 4.8 to 15.4 $\times 10^9$ marrow cells per Kg. and a range of circulating granulocytes of 0.17 to 0.44 $\times 10^9$ cells per Kg. Thus, in contrast to the distribution of erythroid cells where the bulk of the population is in circulation, granulocytic forms of the marrow far outnumber those in active circulation. The ratio between marrow and circulating granulocytic forms was 40:1 in the rabbit, 18:1 in the rat, 35:1 in the monkey and 38:1 in man.

In this marrow population of granulocyte precursors, it is likely that only myelocytes and their precursors are capable of cell division. In man, this represents 2.6 $\times 10^9$ cells. If about one-half of the nucleated erythrocytes are capable of cell division, *i.e.,* the basophilic and polychromatic normoblasts, then the number of mitotable red cell precursors would also represent 2.6 $\times 10^9$ cells per Kg. The presence of mitosis is almost identical in these two series (11). With similar numbers of mitosis in the two cell series, actual production rates will depend on the duration of the mitotic cycle in each.

The supply of mature and near mature granulocytes in the marrow is of interest in leukocyte physiology. Man appears to have a large reserve of mature granulocytes in the marrow—eight times the amount in circulation. However, if both granulocytes and stab forms are included, there are marrow reserves of about 20 times those in circulation. At present, depletion studies by leukophoresis (19) have not removed enough cells to exhaust a reservoir of this capacity; hence, there is no direct evidence that extra-marrow tissue leukocyte reserves exist. The differences obtained from measurements of leukocyte life span may be dependent on whether or not the methods employed include this marrow reservoir (20). Turnover of circulating leukocytes might be expected to be some 10 to 40 times greater than that of combined marrow and circulating leukocyte populations.

TABLE V

Quantitative estimates of hematopoietic tissue of marrow

Author	Species	Erythroid marrow (cells/Kg. $\times 10^9$)	Granulocytic marrow (cells/Kg. $\times 10^9$)
Kindred (6)	Rat	10.0	14.6
Yoffey (8)	Guinea pig	6.7	9.6
Osgood (9)	Man	8.6	25.7*
Patt (10)	Rat	22.0	27.0
	Dog	6.7	11.5
	Man	3.4	8.3
Suit (14)	Man	4.6	
Present report	Rat	5.6	6.7
	Rabbit	5.8	4.8
	Monkey	9.2	15.4
	Man	5.0	11.4

* Exclusive of adult granulocytes.

SUMMARY

Measurements have been made of the cellularity of the erythroid and granulocytic cells in the marrow and circulating blood in three animal species and in man. These data, together with knowledge of rate of red cell production, permit calculation of turnover time of nucleated red cell mass and of marrow reticulocyte pool. While leukocytic production rates and survival data are not available, the figures presented indicate the presence of an appreciable pool of leukocytes in the marrow.

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THE ROLE OF ANAEROBIC METABOLISM IN THE PERFORMANCE OF MILD MUSCULAR WORK. I. RELATIONSHIP TO OXYGEN CONSUMPTION AND CARDIAC OUTPUT, AND THE EFFECT OF CONGESTIVE HEART FAILURE [1]

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Anaerobic energy-yielding processes in human tissues, as estimated from O_2-debt, have been thought to be important in the exertions of athletics (1, 2) or of hard work (3). Recent studies (4) have suggested that the anaerobic metabolism induced by hypoxia plays a part in almost all activity, whether severe or mild, although mild activity may be associated with O_2-debts too small to measure. Rather than an emergency mechanism, anaerobic metabolism may therefore be a part of the ordinary daily life of mammals. This anaerobic mechanism of energy supply is called forth by inadequacy of oxygen supply relative to tissue requirements, and the ability of the oxygen transport mechanisms to meet tissue demands may become the critical factor at specific levels of relative hypoxia (5). Therefore it seemed possible that in patients with diseases of the oxygen transport system (lungs, blood and circulation) tissue anaerobic metabolism might be even more important, and perhaps a significant determinant of physical disability.

The older concept of O_2-debt (6) is a static one largely because of the manner of its measurement, but it has contributed a great deal to the understanding of oxygen supply which could not be learned from blood flow and oxygen concentrations alone. Debt development has been found to be the same in patients with heart failure as in normal persons (7–9). While this might well be regarded as one of the most important facts of the physiology of heart failure, O_2-debt has certain conceptual and practical limitations which will be considered in a later section. However, a workable hypothesis concerning the source of tissue anaerobic energy in man under these circumstances, employing the concept of "excess lactate" (10, 4, 5), permits a more mobile viewpoint which can be extended somewhat beyond the reach of the O_2-debt concept; this notion might be described initially as the instantaneous rate of formation of O_2-debt. On the other hand, if the original static idea is not retained in the terminology, we may conveniently call this notion "anaerobic metabolic rate." If there is a continuous interplay between aerobic and anaerobic metabolism during the daily life of human subjects, as suggested by the apparent occurrence of anaerobic metabolism during slight physical activity, then the quantitative balance between these two phenomena from time to time is of interest both as a physiologic mechanism of work and as a part of the clinical problems of fatigue, dyspnea and physical disability in circulatory disease, problems which have not been satisfactorily understood on the basis of oxygen and blood flow measurements.

The present investigations in normal subjects and in patients with heart disease were undertaken for the purpose of examining the general significance of blood oxygen concentrations, in relation to metabolic adequacy of oxygen supply, by comparing anaerobic and aerobic metabolic rates occurring during mild exercise comparable to the everyday activity of patients.

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[2] Work done in part during tenure of Research Fellowship and Fellowship as Established Investigator of the American Heart Association.

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METHODS

The normal subjects were either hospital patients recovering from minor infectious diseases or patients seeking cardiac or other diagnostic investigation but having no symptoms or other abnormality revealed by the usual diagnostic methods or, in appropriate instances, by cardiac catheterization. The patients in heart failure all had clear-cut disability, *i.e.*, exertional dyspnea, orthopnea, edema, excessive fatigue, although some had only slight complaints. Those with very mild congestive failure (Class II) were included only if these complaints were consistent at various times and could be definitely ascertained to be progressively increasing. All had abnormal hearts on fluoroscopy. Thirteen of the patients exhibited clinical signs of salt retention in the form of edema at the time of the study, but 11 did not. Dyspnea was present in all, however. Six patients had essential hypertension, five had mitral stenosis, two had predominant mitral regurgitation, four had aortic valvular disease and seven had "arteriosclerotic" (senile) heart disease. In the 17 patients studied by cardiac catheterization, pulmonary arterial systolic pressures exceeded 32 mm. Hg and wedge pressures exceeded 18 mm. in every case. Eleven patients (including seven of the nine who had edema) exhibited a right atrial pressure of 10 mm. Hg or above.

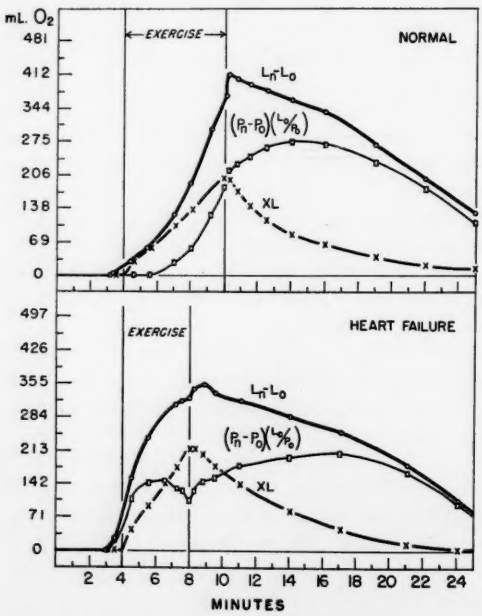

FIG. 1. TYPICAL EXERCISE EXPERIMENTS IN (A) A NORMAL MAN AND (B) A PATIENT WITH CONGESTIVE HEART FAILURE (HYPERTENSIVE, CLASS III)

The pattern of changes in body lactate ($L_n - L_0$), pyruvate $[(P_n - P_0)(L_0/P_0)]$ and excess lactate (XL) with time are shown. Units are milliliters O_2-debt equivalents described in the text. The difference in contours of the time curves of pyruvate change in normal and heart failure subjects illustrates the variations which occur commonly, but either curve (as well as other variations) may be regarded as equally common in both normal and heart failure subjects.

The subjects came to the laboratory fasting. A needle was placed in the brachial artery and in some instances a cardiac catheter was passed into the pulmonary artery, after which the subjects were allowed to rest comfortably for an hour or more. In seven of the normal subjects and five of the patients the metabolic rate at the end of this control period was compared with the basal metabolic rate, as determined on a previous occasion when no other procedure was being carried out, and in no instance was found to differ by more than 5 per cent. Collections of arterial blood were made as previously described (11) with instantaneous denaturation of the blood and with simultaneous determinations of blood water content. Analyses for pyruvate and lactate concentrations were performed as described and expressed as millimoles per liter of blood water. 4-Aminoantipyrine (12) was used to determine total body water content. Determinations of oxygen in arterial blood (Ca_{O_2}) and in mixed venous blood (Cv_{O_2}) were carried out either manometrically or in some instances spectrophotometrically (13) and rates of oxygen consumption ($\dot{V}_{O_2}$) were determined from Haldane analysis of expired air for oxygen, carbon dioxide and nitrogen, and from the volume of air inspired from a calibrated spirometer. The expired air and blood samples were collected simultaneously and continuously over periods of one and one-half to two minutes and cardiac output calculated by the Fick principle. In some instances cardiac output was determined by the dye dilution technique (14) or by both methods. Exercise consisted of a walking motion of the legs at a constant rate while the subject lay supine. Oxygen consumption and cardiac output for the exercise period were determined after five minutes or more of steady activity. This exercise was found to produce a decrease in urinary sodium excretion in patients with congestive heart failure but did not do so in normal man even when continued for 30 minutes.

Concentrations of "excess lactate" (XL) were calculated as described previously (10) from pyruvate and lactate changes, according to the following equation:

$$XL = (L_n - L_0) - (P_n - P_0)(L_0/P_0), \qquad 1)$$

where L_0 and L_n and P_0 and P_n are lactate and pyruvate concentrations in arterial blood water at any two times, t_0 and t_n. In the present experiments t_n was taken as any time during the steady state of exercise and t_0 was taken during the near-basal state of the control period. This calculated fraction of any total lactate change (XL) is attributable to the changes in [DPNH]/[DPN] (diphosphopyridine nucleotide) of tissues rather than in changes in pyruvate, and therefore gives an estimate of the energy derived anaerobically from the lactic dehydrogenase system in response to inadequacy of intracellular oxygen supply relative to cellular energy requirements (10) during

the time interval studied. This energy may be expressed in any of a variety of units, but for the purpose of comparison with rates of oxygen consumption the dimensions used are oxygen-equivalents in milliliters, derived from XL by multiplying by the factor 11.2 ml. of oxygen per mM of XL (4). The total body XL is in turn derived from concentration by multiplying by the volume of body water (4).

RESULTS

Figure 1 shows typical results obtained during mild exercise in a normal subject and in a patient disabled by congestive heart failure (Class III). The observed change in body lactate expressed in oxygen-equivalents (derived from blood lactate concentrations as described above for XL) is shown by the curves marked $L_n - L_0$. No important difference is noticeable between the curves for normal and clinically abnormal subjects; this was a common finding. Two typical forms of the curves are shown, but both types were equally frequent in the two groups of subjects, normal and heart failure. The change in lactate which would be expected from the observed change in pyruvate is shown by the curves marked $(P_n - P_0)(L_0/P_0)$. This much lactate accumulation must be attributed to alterations in pulmonary ventilation, epinephrine secretion, changes in glucose metabolism or other causes (10), but not to deficiency of oxygen supply. The algebraic differences between these two functions at various times is shown by the curve marked XL and represents the excess of lactate accumulation over that which can be accounted for by nonhypoxic causes. The patient illustrated by Figure 1A showed a total XL accumulation of about the same as that of the typical normal subject. The grouped data from 14 normal subjects and from 24 patients with clinical congestive heart failure (disability varying from Class II to Class IV) are shown in the scatter diagrams of Figure 3.

Cardiac output and arteriovenous oxygen difference

Exercise in the normal subjects lasted 8 to 14 minutes, averaging 11 minutes, while that in heart failure patients lasted 5 to 10 minutes, averaging 7.6 minutes. The values for cardiac output, oxygen consumption and arteriovenous oxygen difference at rest, and the changes accompanying exercise, are summarized in Table I for both normal subjects and patients with congestive heart failure. The individual changes of cardiac output ($\dot{Q}a$) and arteriovenous oxygen difference ($Ca_{O_2} - C\bar{v}_{O_2}$) are depicted in Figure 2.

The two groups of subjects differed "significantly" ($p < 0.01$) from each other in both cardiac output and arteriovenous oxygen difference at rest, but the mean increases in cardiac output during exercise did not differ significantly. The

TABLE I

Mean values for measured and derived variables in 14 normal subjects and in 24 patients with heart failure at rest and the changes which occurred on exercise *

Measured and derived variables	Normal		Heart failure			
	Rest	Change with exercise	Rest	†	Change with exercise	†
Time (*min.*)		11 min. (8 to 14)			7.6 min. (5 to 10)	
$\dot{Q}a/M.^2$ (*L./min./M.²*)	3.19 ± 0.29	4.04 ± 2.22	2.50 ± 0.59	<0.01	3.08 ± 1.60	0.05
$Ca_{O_2} - C\bar{v}_{O_2}$ (*ml./L.*)	44.1 ± 5.1	29.8 ± 8.2	56.4 ± 17.0	<0.01	39.0 ± 14.0	0.01
$\Delta\dot{Q}a/\Delta(Ca_{O_2} - C\bar{v}_{O_2})$ (*L./min.*)		6.7 ± 4.1 (4.2–13.3)			5.1 ± 4.3 (0.5–8.6)	0.25
$Paco_2$ (*mm. Hg*)	38 ± 2	+2 ± 4	37 ± 2		−5 ± 4	<0.01
$\dot{V}_{O_2}$ (*ml./min./M.²*)	165.0 ± 45.0	280.0 ± 58.6	167.8 ± 53.4		220.9 ± 91.8	0.02
Lactate (*mM/L.*)	0.620 ± 0.079	1.31 ± 0.060	1.024 ± 0.149	<0.01	2.58 ± 1.06	<0.01
Pyruvate (*mM/L.*)	0.148 ± 0.053	0.090 ± 0.032	0.174 ± 0.098	0.3	0.142 ± 0.144	0.07
L/P	4.24 ± 0.44	4.23 ± 2.51	4.30 ± 0.57		6.52 ± 4.2	0.05
$[XL]_{max.}$ (*mM/L.*)		0.830 ± 0.420			2.370 ± 0.750	<0.01
AMR (*ml./min.*)		26.8 ± 9.96 (4.9–86.0)			129.4 ± 65.3	<0.01
% resp. $\dot{V}_{O_2}$		95.1 ± 1.73 (90–98)			71.2 ± 8.92 (50–80)	<0.01
% resp. $\dot{Q}a$		93.2 ± 1.69			44.7 ± 17.7 (0–69)	<0.01
% resp. $(a - \bar{v})_{O_2}$		92.4 ± 1.71			65.8 ± 11.4	<0.01

* Notations have the form: Mean ± standard deviation (range).

† Indicates the probability of the values in these columns being statistically the same as the corresponding normal values.

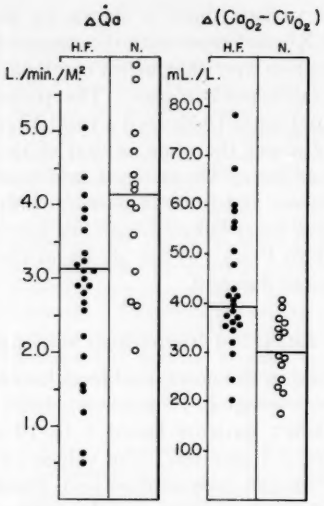

FIG. 2. DIAGRAMS SHOWING THE CHANGES IN CARDIAC OUTPUT ($\Delta\dot{Q}a$) AND IN ARTERIOVENOUS OXYGEN DIFFERENCE $\Delta(Ca_{O_2} - C\bar{v}_{O_2})$ OBSERVED DURING EXERCISE IN NORMAL SUBJECTS (N.) AND IN PATIENTS WITH CLINICAL CONGESTIVE HEART FAILURE (H.F.)

mean changes in cardiac output per liter change in oxygen consumption are also given, but did not differ significantly. Rise of $(Ca_{O_2} - C\bar{v}_{O_2})$ with exercise was higher for the heart failure patients statistically, but many values were within the normal range as shown in Figure 2.

Blood lactate and pyruvate

Blood lactate in the normal subjects increased by a mean of approximately 300 per cent above control. The values in rest and exercise are summarized in Table I. Blood lactate in the heart failure patients increased by about 400 per cent. The mean rise of blood lactate was significantly higher for the heart failure group than for the normals, but the points were widely scattered and overlapped the normal range as shown in Figure 3. The heart failure patients tended to hyperventilate consistently during exercise, raising the oxygen content of expired air and decreasing the carbon dioxide content, whereas the normal subjects uniformly slightly underventilated their lungs relative to $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$. In view of the effect of hyperventilation in producing lactic acidemia (10), most of the difference between the two groups with respect to lactate could be ascribed, in the absence of further information, to a difference in breathing pattern. The rise in arterial blood lactate usually preceded the beginning of exercise, as illustrated in Figures 1 and 6, experiments in which the changes were relatively slight. Increments of blood total lactate on exercise have been found to be particularly striking in subjects noted to be anxious or fearful. Patients in apparently normal physical health who were convinced that they had heart disease (even when they exhibited no overt anxiety) have often shown lactate increases well above the "normal" range while XL responses were quite normal.

The ratio of lactate to pyruvate had about the same mean value in the two groups at rest, and the slightly greater rise of L/P ratio during exercise in the heart failure group was of questionable or no significance as shown in Table I. A tendency toward hyperlactacidemia of arm venous blood in some heart failure patients was recently found by Cotes (15); it had previously been noted in an occasional patient (2, 16) or not found to any significant extent (17, 18), but the validity of methods in the earlier studies is now somewhat questionable. Resting concentrations of lactate in arterial blood shown in Table I averaged significantly higher in the heart failure group than in normal persons. Although the difference in resting pyruvate concentrations is not significant in the sense in which the word has been used here, the difference may be real but more variable.

Excess lactate and anaerobic metabolic rate

As illustrated by the second column of Figure 3 and the data of Table I, XL accumulation gave a sharper separation of the heart failure group from normal. As seen in Figures 1 and 6, and as previously found (4, 5), the curves of excess lactate accumulation were approximately straight lines. The slopes of these lines are shown in the third pair of diagrams of Figure 3, dXL/dt. This value when expressed in milliliters oxygen-equivalents per minute is referred to as the anaerobic metabolic rate (AMR) in each subject for the particular exercise experiment. The detailed calculation of AMR is described in the appendix (A). Anaerobic metabolic rates of the two groups on exercise are shown in Table I.

These figures show a very wide separation of the two groups with almost no overlapping, and appear to be measuring some highly distinctive feature of congestive failure relative to normal.

The fall of XL during recovery after exercise was 87 per cent complete at 30 minutes in the heart failure patients, about the same as in the normals (average, 93 per cent completion in 30 minutes). Both were very variable.

Correction for variations in severity of effort

AMR, like most physiologic measurements which change during exercise, is greater with severe exertion and less with mild exertion (4). A simple difference in severity of exercise, then, might be the only difference between these two groups of subjects, and the heart failure patients may actually have been performing a greater amount of "internal work" (*i.e.*, expending more energy) to account for these results, rather than exhibiting any more direct effect of the circulatory disease. This concept of internal work was referred to by Furusawa, Hill, Long and Lupton (19) as "effort," as opposed to the mechanical work (measured externally) to which it bore no simple or predictable relationship in their studies (20), even in normal subjects, nor in any one subject at different times.

Severity of effort has sometimes been estimated from the rise in rate of oxygen consumption. There is, however, clear evidence in the present data to suggest that, in the patients with heart failure, oxygen consumption did not account for the energy expenditure of exercise, since the anaerobic metabolic rate was markedly increased.

The estimate needed for this correction is the "total metabolic rate" for each exercise, or the energy cost of the effort. Total metabolic rate (TMR) was therefore calculated for each experiment as the sum of the aerobic metabolic rate of exercise ($\Delta\dot{V}_{O_2}$) plus the anaerobic metabolic rate. The AMR in each instance could then be expressed per unit of TMR, that is, AMR/TMR. The bar graphs of Figure 4A show by total height the TMR in each case; amounts of exertion were obviously very variable in both groups. In section B each individual TMR is set at 100 per cent and the $\Delta\dot{V}_{O_2}$ and AMR in each case are illustrated as fractions of the total. AMR,

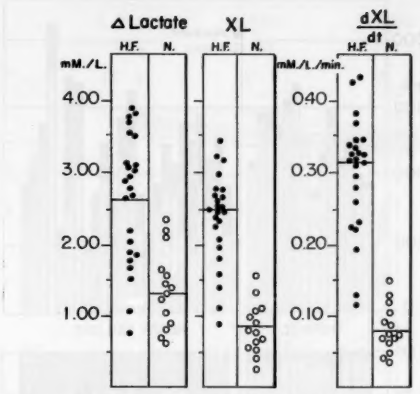

FIG. 3. DIAGRAMS OF THE DATA OBTAINED FROM EXERCISE EXPERIMENTS IN 14 NORMAL SUBJECTS (N.) AND 24 PATIENTS WITH CONGESTIVE HEART FAILURE (H.F.)

The maximum change in blood lactate and excess lactate are shown in the first and second columns, respectively. Unlike $\dot{V}_{O_2}$, $\dot{Q}a$ and $(Ca_{O_2} - C\bar{v}_{O_2})$, which are constant after the first two minutes of exercise, these values change continuously and the exact timing of the measurement period is important; dXL/dt is shown in the right hand column.

the upper sections, is seen to supply about 5 per cent of total energy requirement in the normal subjects and 20 to 50 per cent in the cardiac patients.

Percentage response of oxygen consumption

The same information which is provided by dividing AMR by TMR will also be given by dividing $\Delta\dot{V}_{O_2}$ by TMR, since this is simply (1 − AMR/TMR); but the resulting value (the lower sections of the bars in Figure 4B) proves to be particularly useful. This latter figure represents the amount of response of overall oxygen delivery as a fraction of total energy requirement, *i.e.*, oxygen consumption as a percentage of what it would have been had it supplied the total energy needs of the body. This value will be referred to as "percentage response of oxygen consumption" (% resp. $\dot{V}_{O_2}$). The calculation is as follows:

$$\%\ \text{resp.}\ \dot{V}_{O_2} = \frac{\Delta\dot{V}_{O_2} \times 100}{\text{AMR} + \Delta\dot{V}_{O_2}}. \qquad 2)$$

The anaerobic metabolic rate and change in oxygen consumption rate ($\Delta\dot{V}_{O_2}$) are those simultaneously observed during a given change from

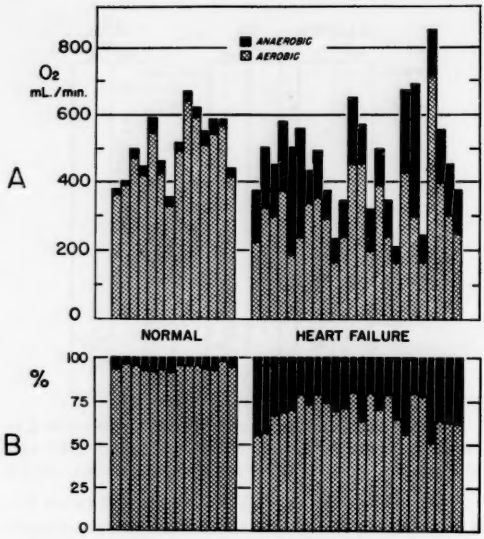

FIG. 4. RELATIONSHIPS OF AEROBIC AND ANAEROBIC METABOLIC RATES IN NORMAL SUBJECTS AND PATIENTS WITH HEART FAILURE

A. The length of the hatched bars denotes the magnitude of change in oxygen consumption for each exercise experiment and the length of each black bar denotes the magnitude of the anaerobic metabolic rate found in the same experiment. The total height of each column therefore shows the total energy requirement or total metabolic rate (TMR) of each exercise.

B. The data from section A are adjusted to make each TMR equal 100 per cent. The lengths of hatched and black bars therefore denote, respectively, change in oxygen consumption and AMR as per cent of TMR for each experiment, and permit comparison of experiments of different degrees of exertion with each other.

rest to exercise. The detailed calculation is shown in the appendix (B).

This correction for differences in effort performed by each subject did not reduce the difference between the two groups of data, but on the contrary accomplished a complete separation. Normal subjects were able to supply 95.1 per cent of body oxygen requirements at the time of the need as molecular oxygen, *i.e.*, by cardiorespiratory response. Patients with heart failure were able to supply only 50 to 80 per cent of requirement. There was no overlapping of the data, and in fact no values between 80 and 90 per cent occurred. This expression therefore appeared to measure some highly characteristic feature of heart failure.

The data for these calculations are all referred to differences between one time, t_n, and another, t_0; the former is taken to be any time during a mild exercise in these experiments, while the latter is taken as the near-basal state previously described (4). However, this experimental design is apparently not critical to the results since, as will be suggested by experiments described below, any two times in the life of the subject would probably serve equally well to reveal the percentage response of oxygen consumption, provided only that changes occurred which were measurable by the techniques employed, and that the changes were produced by exercise.

Percentage response of cardiac output

The findings which distinguish between the two groups of subjects appear to be characteristic of heart failure, the chief clinical difference between them, but failure of overall oxygen delivery could presumably be due to impairment of aspects of oxygen transport other than circulatory. Calculation of a "percentage response of cardiac output" therefore might indicate that the response of the heart in the patient group was not actually at fault. This value was obtained by calculating the "cardiac output deficit," an amount of additional cardiac output which would have been required to supply the extra oxygen needed to prevent the observed anaerobic metabolic rate, all other factors being the same:

$$\text{cardiac output deficit} = \frac{\text{AMR}}{(Ca_{O_2} - C\bar{v}_{O_2})_{\text{exercise (ex.)}}}.$$

The percentage response of cardiac output (% resp. $\dot{Q}a$)[4] then, in terms of the measured quantities, is:

$$\%\ \text{resp.}\ \dot{Q}a = \frac{\Delta\dot{Q}a \times 100}{\Delta\dot{Q}a + \text{deficit}} \qquad 3)$$

$$= \frac{\Delta\dot{Q}a \times 100}{\Delta\dot{Q}a + \dfrac{\text{AMR}}{(Ca_{O_2} - C\bar{v}_{O_2})_{ex.}}}.$$

In the same manner a "percentage response

[4] The symbol $\dot{Q}a$ (volume flow of arterial blood per unit time) (21) is used to denote cardiac output in the sense of rate of total peripheral blood flow through the body.

of arteriovenous oxygen difference" [% resp. $(a - \bar{v})_{O_2}$] may be calculated:

$$\%\ \text{resp.}\ (a - \bar{v})_{O_2} = \frac{\Delta(Ca_{O_2} - C\bar{v}_{O_2}) \times 100}{\Delta(Ca_{O_2} - C\bar{v}_{O_2}) + \dfrac{AMR}{\dot{Q}a_{ex.}}} \quad 4)$$

The detailed calculation of these percentage responses is shown in the appendix (C). Figure 5 shows the calculated values plotted in scatter diagrams for the normal subjects and for the patients. Statistical descriptions are given in Table I. Points in the first pair of columns give the same values as the lower bars of Figure 4B, the % resp. $\dot{V}_{O_2}$. The values of the three percentage responses found for a given subject at one time are joined by lines to show the individual relationships. It is clear from the figure that percentage responses of cardiac output in heart failure did not prove to be nearer to normal values than was the adequacy of gross oxygen supply rate, but were more abnormal in every case.

While the lowest value of % resp. $\dot{Q}a$ in Figure 5 was for a patient with Class IV disability and the highest for a patient with Class II disability, a close correspondence was otherwise not noted between the figures and independent estimates of clinical severity. All classes, II, III and IV, were represented in the group of points between the extremes, as indicated by the numerals adjacent to the points in Figure 5.

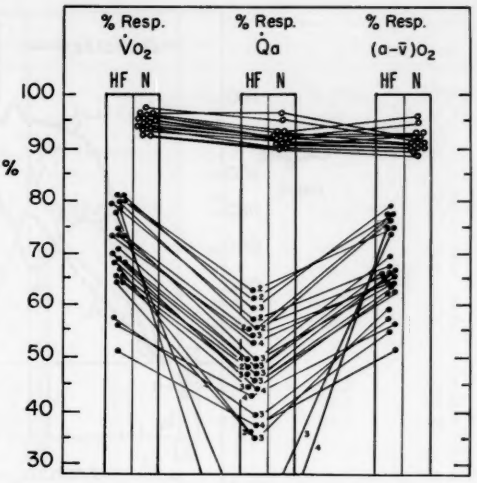

FIG. 5. PERCENTAGE RESPONSES OF OXYGEN CONSUMPTION (% RESP. $\dot{V}_{O_2}$), CARDIAC OUTPUT (% RESP. $\dot{Q}a$), AND ARTERIOVENOUS OXYGEN DIFFERENCE [% RESP. $(a - \bar{v})_{O_2}$] IN NORMAL (N.) AND HEART FAILURE (H.F.) SUBJECTS

Each line joins the three values obtained simultaneously in a given exercise experiment. The small numeral adjacent to each point denoting % resp. $\dot{Q}a$ of a heart failure patient gives the clinical classification of disability assigned prior to the experiment. A division of the points by etiology yielded no systematic differences and is therefore not given here, but the three lowest points represent patients with predominant mitral regurgitation.

Reproducibility of percentage responses

The reproducibility of the derived figures is illustrated in Figure 6. The same subject underwent two different exercise experiments of differing severity. None of the measured changes was the same in Experiment B as in Experiment A. The percentage responses, however, were almost identical in the two experiments. Three normal subjects had been studied in this way in repeated experiments which differed in severity of exertion. The percentage responses of oxygen consumption were duplicated with errors of less than 3.5 per cent as shown in Table II.

In Figure 6B are also indicated two separate times of complete sampling in a single period of continuous exercise; the times are marked $t_{n'}$ and t_n. The percentage response values given at the top of Figure 6B and in Table II (B) are those for the entire exercise period, as they were reported for all the other experiments. However, when values obtained at time $t_{n'}$ are compared with controls in computing percentage responses, about the same results are obtained. Of more fundamental importance, on the other hand, is the similarity of percentage responses derived from comparison of sampling times t_n and $t_{n'}$ for the calculation, since these are two times during exercise, without any reference to the basal state.

Table II (C) also shows the values of the percentage responses in replicate studies of four patients with congestive heart failure who originally showed various degrees of abnormality. Again the derived values were reproducible within about 3 per cent. These derived values therefore appear to represent an interrelationship which is characteristic of the individual and is

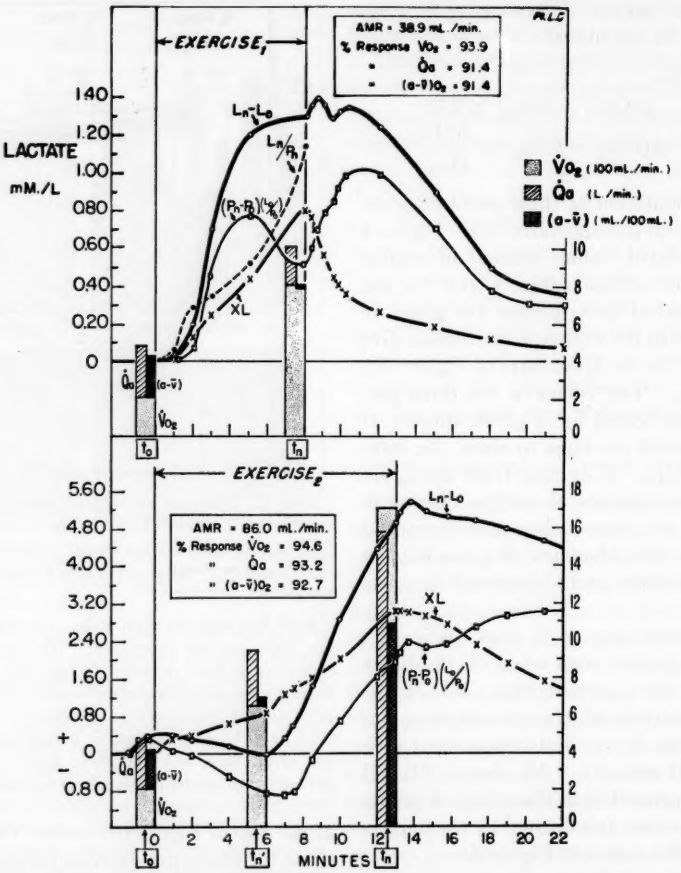

FIG. 6. TWO EXERCISE EXPERIMENTS CONDUCTED ON ONE NORMAL SUBJECT SEVEN DAYS APART

Experiment B utilized more severe exertion and exhibited a greater rise in oxygen consumption ($\dot{V}_{O_2}$), cardiac output ($\dot{Q}a$), arteriovenous oxygen difference ($a - \bar{v}$), blood lactate ($L_n - L_0$), blood pyruvate $[(P_n - P_0)(L_0/P_0)]$, and excess lactate (XL) as well as a larger anaerobic metabolic rate (AMR). Percentage responses were the same in the experiments, however. In addition to the comparison of the whole exercise periods A and B, percentage responses can be compared in the two parts of exercise B ($t_n + t_{n'}$), and again are approximately the same.

largely independent of the severity and duration of effort (at least within the limits studied) and of the absolute values of the circulatory and respiratory responses to the effort, as well as being independent of the levels from which these responses begin.

The responses of normal persons do not approach those of heart failure patients as exercise becomes increasingly severe in the normal subject and less severe in the patient. The difference between them appears to be a difference in fundamental mechanism of energy supply.

DISCUSSION

The objective of the present experiments was to evaluate further the physiologic significance of

TABLE II

Reproducibility of percentage responses in selected subjects

Subject	$\Delta\dot{V}_{O_2}$	$\Delta\dot{Q}a$	$(Ca_{O_2} - C\bar{v}_{O_2})_{ex.}$	AMR	% resp. $\dot{V}_{O_2}$	% resp. $\dot{Q}a$	% resp. $(a - \bar{v})_{O_2}$
	ml./min.	*L./min.*	*ml./L.*	*ml./min.*			
A. Normal							
1 (a)	460	4.12	78.6	26.1	94.5	92.5	93.2
1 (b) (same day)	690	4.84	93.0	36.0	95.0	92.0	93.7
2 (a)	665	6.83	76.5	33.9	95.1	93.9	95.7
2 (b) (after 8 days)	325	2.14	69.0	6.0	98.3	96.1	97.1
3 (a)	280	2.90	82.0	21.0	93.1	91.9	92.2
3 (b) (after 7 days)	897	4.66	104.2	54.0	94.3	90.0	94.2
B. Normal (Fig. 6B)							
Total period	1,510	10.60	110.0	86.0	94.6	93.2	92.7
$t_{n'}$ − control	450	5.00	71.1	26.0	94.3	93.4	92.0
$t_n - t_{n'}$	1,060	5.60	110.0	60.0	94.7	91.2	90.9
C. Heart Failure							
1 (a) Class II, A.S., A.R.	250	1.00	114.1	97.0	72.0	54.1	70.3
1 (b) (same day)	375	1.46	123.2	154.2	70.9	53.9	69.2
2 (a) Class III, hypertension	400	1.24	108.5	129.2	75.8	51.0	73.1
2 (b) (same day)	270	1.01	94.3	99.4	73.2	48.8	71.4
2 (c) (after 7 days)	620	1.62	116.2	194.7	76.1	49.2	72.0
3 (a) Class III, A.S.H.D.	375	1.81	89.7	240.0	61.0	40.3	59.0
3 (b) (after 7 days)	680	2.16	120.0	400.8	62.9	39.4	60.4
4 (a) Class II, hypertension	410	1.26	86.0	171.3	70.6	38.9	66.7
4 (b) (same day)	190	0.70	78.9	90.2	67.8	38.0	64.7

blood oxygen concentrations and of oxygen exchanges. The phenomena of exercise and of heart failure both present peculiarly complex problems with regard to the interpretation of such measurements. The metabolic evaluation undertaken consisted of two parts: the measurement of rates of anaerobic metabolism during mild exercise in normal subjects and in patients with heart failure, and the examination of the relationships between anaerobic metabolic rate and aerobic metabolic rate or oxygen consumption (with its component parts, cardiac output and arteriovenous oxygen difference). While the simplest expression of relationship would perhaps be the ratio of oxygen consumption to AMR, a value which, like the "percentage response of oxygen consumption," would be self-corrected for body size and for severity of exertion, nevertheless this is an arbitrary index. Although empirical significance could be assigned to such values with experience, this procedure would not actually represent a gain in simplicity. The "percentage response of cardiac output" is somewhat more complex in calculation, but it is analogous in concept to the percentage response of oxygen consumption. These expressions of interrelationship should not be interpreted as implying interdependence of their elements, at least not directly.

A. Normal subjects

In normal human subjects during ordinary daily life the balance between the AMR and $\dot{V}_{O_2}$ of tissues appears to be variable, but is such that 95 ± 1.7 per cent of the energy needs of the body are supplied at the time of the need by circulatory-respiratory response. Thus a very high percentage of the energy needs is met by the oxygen transport mechanism, but the normal response is not perfect. The role played by these

small rates of anaerobic metabolism in slight muscular activity remains to be elucidated. Hill, Long and Lupton (1) have discussed the possibility that the O_2-debt[5] development of severe exercise permits a degree of exertion to which oxygen supply from outside the cell probably could not adjust instantaneously. Millikan's demonstration that intracellular P_{O_2} falls markedly with each muscle contraction offers support to this idea (22). The same principle can perhaps also be applied to nonemergency activity like that of the present experiments.

The form of the time curves of excess lactate in both groups of subjects appears to be approximately a straight line, *i.e.*, during the exercise periods of these studies, dXL/dt was roughly constant, suggesting that anaerobic metabolic rate not only was independent of lactate and pyruvate concentrations, which changed irregularly, but also was a zero order process and independent of XL concentration itself. The lactate-pyruvate ratio altered continuously and often irregularly during exercise, as illustrated in Figure 6A, while AMR remained constant. L/P ratio, however, is presumably affected by hydrogen ion concentration, temperature and ionic strength of the cellular medium as well as by other influences.

B. Effects of circulatory disease

Whatever may be the function of small rates of anaerobic metabolism in tissues, or the method of their control in the normal organism, heart failure brings the anaerobic metabolism of patients into much greater prominence. It seems possible that some patients are dependent on this physiologic mechanism for the slightest activity. This new balance between aerobic and anaerobic metabolism was a unique difference from normal subjects in the present groups.

In view of this finding with respect to AMR, it may seem surprising that data available in the literature on O_2-debt estimations in heart failure, when examined statistically, fail to demonstrate alteration of the normal relationship between O_2-debt development and severity of exertion (7–9, 23). Herbst (9), for example, found a difference between the O_2-debt development of young athletes doing maximal running exercise and that of various groups of patients who performed a moderate arm exercise; but patients with heart failure were not found to differ from those who had no heart failure, nor did they differ from a group of nervous individuals. It was shown that cardiac decompensation could not account for any metabolic differences among individuals in data published up to that time. These findings are in agreement with observations on O_2-debt made in connection with the present experiments. Several writers have expressed disagreement with this idea (18, 24, 25) but unfortunately have given no data. It might be hoped that Nylin's "cardio-pulmonary function test" (26) would show some correlation with debt; however, analysis of a number of O_2-debt studies in this laboratory has revealed no correlation. In any event, other studies of this respiratory test (27) have failed to show a significant difference between normal subjects and patients with heart failure alone.

These various findings may seem difficult to reconcile with the present data, but the technique of measurement of O_2-debt is very exacting (1, 4), and the results may be affected by many extraneous factors. Compromises in method introduced by some experimenters have no doubt increased the large random variance always found. Moreover, short bursts of strenuous exercise have usually been employed. Fairly severe exercise is essential for acceptable accuracy in O_2-debt measurement, but such unsteady states with respect to respiration and diffusion of gases in the lungs and elsewhere are far more complex than those of the present experiments. The factors affecting the emergency role of anaerobic metabolism are less clear, and require further investigation.

1. *Significance of "total" lactate.* There appeared to be some tendency for absolute blood lactate concentration to increase more in about half the patients with heart failure than in the normal subjects. While blood total lactate concentrations were once believed to be related to oxygen deficiency (16) because of a hypothetical association with O_2-debt, this idea is no longer quite tenable. The causes of nonhypoxic lactic acidemia in either group are probably multiple.

[5] Theoretically, $O_2\text{-debt} = \int_{t_0}^{t_n} (AMR)dt$, and in practice the approximation is good when conditions are ideal for the respiratory measurements (4).

Hyperventilation, one of the most potent causes (10), may be a significant element in heart failure, since some of these patients characteristically hyperventilate during exercise (7, 9, 15). Part of the variations in normal subjects, as well as the effects of heart failure itself, may be due to some influence of sedentary living, a factor which may significantly increase lactate response, although not XL responses, to exercise. Physical training reduces the lactate response to exercise (28, 29, 4), but this likewise is often due merely to decrease of pyruvate response (4) instead of reduction of XL accumulation, and thus is frequently not due to improvement of oxygen delivery with training. However, the explanation for reduction of total lactate response by training may be its effect on ventilatory response, since physical fitness appears to cause an increased carbon dioxide retention in exercise (28).

2. *Significance of blood oxygen.* In severe congestive heart failure the oxygen content of mixed venous blood falls lower during exercise than the average in a group of normal subjects. This fact might have meaning if there were assurance that the internal work of doing the exercise was the same in the two groups; but, even so, the meaning in terms of tissue metabolic effects would not by any means be clear. The partial substitution of increased blood oxygen extraction for increase of blood flow is a normal mechanism of oxygen delivery and such decreases in venous blood oxygen usually occur with increased rates of delivery of oxygen, as observed in the present normal subjects, rather than being associated with decreased oxygen delivery. The intracellular P_{O_2} which is critical to oxygen supply cannot be determined from gross oxygen data alone, as previously pointed out (5). There has been no evidence that the $C\bar{v}_{O_2}$ in patients with heart failure represents or causes a deprivation of oxygen to the body relative to its needs. When similar blood oxygen changes occur with the breathing of low oxygen gases, for example, the rate of supply of oxygen per minute to body tissues does not diminish, and there may be no O_2-debt formed (5). A more accurate point of view with respect to oxygen concentrations seems to be that some heart failure patients may deliver oxygen to the tissues in a different manner than some normal subjects.

3. *Anaerobic metabolism as a third adaptive mechanism in oxygen lack.* Both increased blood oxygen extraction and increased blood flow normally occur together as the need for increased oxygen delivery augments. Anaerobic tissue metabolism appears to join these changes as a third adjustment to increased oxygen need, not when the first two have become maximal, but before this. In neither the normal subjects nor the patients with heart failure was the exercise near maximal. This principle is opposed to the previous belief that "the oxygen demand or requirement of the exercise is met until the oxygen intake is at a maximum" (18). Studies of severe or "maximal" exercise (7, 9, 18) have been of great interest in some respects, but the results in cardiac patients are those which would have been expected if the patients had been normal subjects who chose to exercise less than the others, but who were otherwise similar.

The concept of a "100 per cent response" as employed here, *i.e.*, what the oxygen supply or cardiac output "should" be, is a theoretical value; it is nevertheless derived for a particular individual at a particular instant, and is not of the nature of a "normal" value obtained from other subjects for reference. Normal subjects do not achieve 100 per cent response. This aspect of the calculation represents its fundamental difference from changes in oxygen consumption or cardiac output *per se*, or changes from some mean value obtained in other individuals. The 100 per cent response value is dependent on the measurement of dXL/dt, or AMR, at the desired moment in the subject who is being examined. The statistically "normal" values of the mean $\dot{Q}a$ and $\dot{V}_{O_2}$ responses in the present patients clearly do not preclude the finding of low percentage responses and, in addition, this kind of cardiac output deficit may be present when changes of arteriovenous oxygen difference also are entirely "normal" statistically (30). On the other hand, the percentage response of oxygen consumption may be normal in certain subjects with abnormally reduced % resp. $\dot{Q}a$ (30).

The reverse is not true, however. "Percentage response of cardiac output" has as its practical upper limit the value of % resp. $\dot{V}_{O_2}$. From this characteristic it follows that depression of % resp. $\dot{Q}a$ would also be expected to occur in

any other condition which depressed % resp. $\dot{V}_{O_2}$, even when the performance of the heart was normal. However, a depression of % resp. $\dot{Q}a$ in excess of that occurring in % resp. $\dot{V}_{O_2}$ would not be expected. Preliminary experience has shown that severe anemia and pulmonary disease depress the % resp. $\dot{V}_{O_2}$ in some instances to values between 80 and 90 per cent, but when this happens the % resp. $\dot{Q}a$ is numerically the same, in distinct contrast to the effect of congestive heart failure. These findings will be presented in detail at a later time.

The physiologic significance of the relationship between rate of tissue anaerobic metabolism and rate of blood flow is clarified somewhat further by observations on the effect of relatively slight obstructions to blood flow in the absence of heart failure (30).

4. *"Inadequacy" of oxygen delivery and "insufficiency" of the circulation.* Theories of the basic circulatory defect of congestive heart failure as an "inadequacy" of cardiac output have been repeatedly advanced (31–33) and denied (34). Fundamental objections to such a concept have remained; these physiological difficulties have, in effect, been discussed in the preceding sections.

In the more practical sphere, certain observations in heart failure are also difficult to explain on the basis of a "forward failure" concept. Patients with pulmonary disease (35) and anemia (36) at rest have the same $C\bar{v}_{O_2}$ when in heart failure as before failure occurs. $P\bar{v}_{O_2}$ falls lower in many anemic patients who have compensated hearts than is ever found in ordinary patients with decompensation (37). Further reduction of blood oxygen does not appear to accentuate progression of the syndrome of heart failure as estimated from sodium retention (38). Moreover, patients with heart failure due to common cardiac diseases often have no lower cardiac output or higher arteriovenous oxygen difference than patients who are clinically compensated after once having been in failure (39). Aside from these aspects of blood flow and oxygen concentration, the O_2-debts developed by patients with heart failure cannot be shown to differ from normal, at least with strenuous exertion. And finally, evidence has been presented that such equivocal blood lactic acid changes as have been described have no bearing on the question of adequacy of oxygen supply (4, 5, 10). We have not been able to substantiate our previous belief that "for any given level of anxiety or work" the patient with congestive failure has an arteriovenous oxygen difference greater than normal (33). This belief has been based largely on the idea that the "level of anxiety or work" can be judged from the rate of consumption of oxygen, an error which has been pointed out in the past for severe exertion (1) and requires no further emphasis here than to present the anaerobic metabolic rates found in mild exercise in the present series of patients. The present results, nevertheless, support the "forward failure" hypothesis concerning the fundamental derangement in congestive heart failure in suggesting that the rate of supply of oxygen to the sites of its metabolism in the tissues is inadequate to meet actual metabolic requirements in patients with congestive heart failure during even mild activity, and that this inadequacy is specifically due to inadequacy of response of the heart on demand.

The question of the physiologic significance of blood oxygen content or tension extends beyond the problem of the pathogenesis of congestive heart failure. The same question also arises in considering problems of regional ischemia and of anemia, pulmonary disease and high altitude effects (5), among others. It would appear to be necessary in such situations to use the greatest caution in assigning any significance to arterial or venous blood oxygen values as indications of oxygen deficiency relative to the needs of the tissues.

SUMMARY

1. A progressive accumulation of "excess lactate" occurred in normal human subjects during the performance of muscular work so mild as to be comparable to ordinary daily activity, a finding which indicated a shift in the mean steady-state position of the total body lactic dehydrogenase system toward the reduced state.

2. The energy derived from this anaerobic metabolism in normal persons varied widely in absolute magnitude but constituted about 5 per cent of total energy utilization in the various grades of exercise studied, suggesting that oxygen transport to tissues was 95 per cent effective in

meeting body requirements despite decreases in mixed venous blood oxygen.

3. During similar exercise, all patients with clinical heart failure exhibited rates of anaerobic metabolism greater than those observed in normal subjects, oxygen transport being only 50 to 80 per cent effective in meeting energy requirements, regardless of whether mixed venous blood oxygen was "normal" or low.

4. The fraction of total energy requirement which could be met by oxygen transport in an individual under varying degrees of stress was a characteristic of the individual (and not of the magnitude of stress) both in normal subjects and in patients with heart failure.

5. Cardiac output responses in exercise throughout the range studied bore a predictable relationship to rates of anaerobic metabolism in normal subjects such that a further increase in cardiac output of about 7 per cent would have supplied the oxygen need which was manifested as anaerobic metabolism.

6. Exercise responses of cardiac output in the patients with heart failure were not significantly different from normal, but the relationship to arteriovenous oxygen difference and anaerobic metabolism was such that an increase of 40 to 90 per cent would have been required to supply the additional oxygen requirement of the body. This fraction invariably exceeded the fractional deficit of overall oxygen delivery, and thus exhibited a relationship which appeared to be a unique characteristic of heart failure.

7. It is concluded that: *a*) Rates of oxygen consumption do not accurately measure the degree of exertion in human subjects performing work, and the error is systematically greater in patients with circulatory diseases than in normal subjects. *b*) Estimates of molecular oxygen concentrations in body fluids, or their rates of exchange do not serve to indicate whether oxygen supply is adequate to meet the tissue requirements of the moment. *c*) A basic physiologic derangement in all patients with congestive heart failure is an inadequacy of oxygen supply rates to meet tissue energy requirements, an inadequacy which is specifically due to insufficiency of cardiac response, but which is neither indicated nor measured by the observed changes in oxygen delivery or cardiac output.

APPENDIX

A. Table III shows the observed data in one normal, a 32 year old woman, and one abnormal subject, a 54 year old man with hypertension and Class III congestive heart failure. From Line 2, the changes in lactate concentration with exercise ($L_n - L_0$) were 1.252 mM per L. and 3.875 mM per L. for the normal subject and the patient, respectively. The changes in pyruvate concentration ($P_n - P_0$), Line 3, were 0.091 mM per L. and 0.156 mM per L. The ratios of resting lactate and pyruvate concentrations (L_0/P_0) (Lines 2 and 3) were 3.91 and 5.73. Substituting in Equation 1

$$XL = (L_n - L_0) - (P_n - P_0)(L_0/P_0) \qquad 1)$$

gives final XL concentrations of 0.896 mM per L. in the normal subject and 3.939 mM per L. in the patient with heart disease. XL concentration per liter of water is multiplied by the volume of water in the body, W_b, for each subject (Line 4) to give total XL accumulations of 27.0 and 116.6 millimoles. From the durations of exercise in Line 1, the rates of accumulation are shown to be 2.70 mM per minute and 12.96 mM per minute. The oxygen equivalent of each millimole of XL is taken to be 11.2 ml., giving AMR values of 30.2 ml. per minute and 145.0 ml. per minute, respectively. These data illustrate the occurence of high blood lactate with high AMR. However, Section C gives an example of very marked differences in AMR in two subjects with normal lactate changes.

TABLE III

Observed data from experiments on one normal subject and one subject with congestive heart failure, showing calculation of excess lactate (XL) and anaerobic metabolic rate (AMR)

	Normal (female, 32 yrs.)		Heart failure (male, 54 yrs.)	
	Rest	Exercise	Rest	Exercise
Duration (*min.*)		10.0		9.0
Lactate (*mM/L.*)	0.614	1.866	0.956	4.831
Pyruvate (*mM/L.*)	0.157	0.248	0.167	0.323
Weight (*Kg.*)	61.6		54.6	
W_b (*L.*)	30.1		29.6	
XL (*mM/L.*)		0.896		3.939
AMR (*ml./min.*)		30.2		145.0

TABLE IV

Observed data from experiments on one normal subject and one subject with congestive heart failure, showing calculation of percentage response of oxygen consumption (% resp. $\dot{V}_{O_2}$)

	Normal (female, 52 yrs.)		Heart failure (male, 36 yrs.)*	
	Rest	Exercise	Rest	Exercise
Duration (*min.*)		7.0		8.0
Lactate (*mM/L.*)	0.550	1.186	0.870	3.638
Pyruvate (*mM/L.*)	0.135	0.220	0.205	0.406
Weight (*Kg.*)	52.0		72.0	
Surface area ($M.^2$)	1.51		2.04	
W_b (*L.*)	24.5		39.4	
$\dot{V}_{O_2}$ (*ml./min.*)	196.5	399.0	365.0	853.0
$\dot{V}_{O_2}$ (*ml./min./M.²*)	130.0	264.0	179.0	418.0
$\dot{V}$ (*ml./min.*)	5,150.0	1,020.0	7,920.0	21,300.0
XL (*mM/L.*)		0.290		2.785
AMR (*ml./min.*)		11.4		153.6
% resp. V_{O_2} (%)		94.7		76.0

* Protocols illustrate the occurrence of a low % resp. $\dot{V}_{O_2}$ in a patient whose increase in $\dot{V}_{O_2}$ was apparently normal.

B. Table IV gives the original data obtained from one normal subject and a patient with congestive heart failure (Class II, mitral stenosis and regurgitation, edema for one year). Substituting, as in A, in the composite equation

$$AMR = \frac{11.2\, W_b[(L_n - L_0) - (P_n - P_0)(L_0/P_0)]}{t} \quad 5)$$

AMR values of 11.4 ml. per minute and 153.6 ml. per minute are obtained in the two subjects. The changes in oxygen consumption for the experiments ($\Delta\dot{V}_{O_2}$), Line 7, were 202.5 ml. per minute for the normal subject and 488 ml. per minute for the patient. Substituting in Equation 2

$$\%\ \text{resp.}\ \dot{V}_{O_2} = \frac{\Delta\dot{V}_{O_2}}{AMR + \Delta\dot{V}_{O_2}} \quad 2)$$

gives percentage responses of oxygen consumption of 95.0 per cent and 76.0 per cent for the normal subject and the

TABLE V

Observed data from experiments on one normal subject and one subject with congestive heart failure, showing calculation of percentage responses of oxygen consumption and cardiac output

	Normal (female, 39 yrs.)		Heart failure (female, 64 yrs.)*	
	Rest	Exercise	Rest	Exercise
Duration (*min.*)		5.0		5.0
Lactate (*mM/L.*)	0.760	2.822	0.714	3.163
Pyruvate (*mM/L.*)	0.159	0.311	0.138	0.210
W_b (*L.*)	24.3		38.0	
Surface area ($M.^2$)	1.44		1.90	
$\dot{V}_{O_2}$ (*ml./min.*)	198.0	836.0	351.0	1,208.0
$\dot{V}$ (*ml./min.*)	5,350.0	22,000.0	8,450.0	14,050.0
$\dot{Q}a$ (*L./min.*)	4.83	10.60	8.07	11.50
$Ca_{O_2} - C\bar{v}_{O_2}$ (*ml./L.*)	41.0	78.9	43.5	105.0
RA (*mm. Hg*)	4.0		11.0	
PA (*mm. Hg*)	16/7	17/7	34/19	70/42
PC (*mm. Hg*)	5		8	25
XL (*mM/L.*)		0.643		2.790
dXL/dt (*mM/L./min.*)		0.129		0.558
AMR (*ml./min.*)		35.0		237.5
% resp. $\dot{V}_{O_2}$ (%)		94.8		74.0
% resp. $\dot{Q}a$ (%)		92.9		60.3
% resp. $(a - \bar{v})_{O_2}$ (%)		92.0		74.9

* The heart failure subject is an example of one who showed "normal" blood lactate and arteriovenous oxygen differences at rest and after exercise. Although the patient's $\Delta\dot{Q}a$ is less than that of this particular normal subject, it is about the same as that seen in other normal subjects.

patient, respectively. Both oxygen consumption values are, however, well within the normal range.

C. In Table V are given the original data obtained in an additional normal subject and in another patient with heart failure (Class III, 64 years, senile heart disease). Calculating AMR as in A and B gives the values 35.0 ml. per minute and 237.5 ml. per minute, respectively. Calculating % resp. $\dot{V}_{O_2}$ as in B gives 94.8 per cent and 74.0 per cent for the two subjects. From Line 8 it can be seen that cardiac output increased by 5.77 L. per minute in the normal subject during exercise ($\Delta\dot{Q}a$), and increased by 3.43 L. per minute in the patient with clinical heart failure. Moreover (Line 9), the final exercise values for arteriovenous oxygen difference $(Ca_{O_2} - C\bar{v}_{O_2})_{ex.}$ were 78.9 ml. per L. and 105.0 ml. per L., respectively. Substituting in Equation 3,

$$\%\ \text{resp.}\ \dot{Q}a = \frac{\Delta\dot{Q}a}{\Delta\dot{Q}a + \dfrac{AMR}{(Ca_{O_2} - C\bar{v}_{O_2})_{ex.}}} \qquad 3)$$

the percentage responses of cardiac output are found to be 92.9 per cent for this normal subject and 60.3 per cent for the patient. By a similar substitution from Table V in Equation 4, the percentage responses of arteriovenous oxygen difference are found to be 92.0 per cent and 74.9 per cent, respectively. The data shown in Table V for the patient with heart failure give an example of normal cardiac output change with exercise, by our standards, but the percentage responses are quite low. Although the arteriovenous oxygen difference seems somewhat high, some normal subjects exhibit equally high values during exercise.

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THE ROLE OF ANAEROBIC METABOLISM IN THE PERFORMANCE OF MILD MUSCULAR WORK. II. THE EFFECT OF ASYMPTOMATIC HEART DISEASE[1]

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Previous studies of the anaerobic metabolism called forth by oxygen lack appeared to suggest that clinically apparent congestive heart failure is always associated with an inordinate degree of tissue anaerobic metabolism and therefore an inadequate delivery of oxygen to body tissues during mild muscular activity (1). Moreover, the data implied that this restriction, in the case of heart failure, was due to inadequacy of cardiac output response and not to failure of other portions of the general oxygen transport mechanism. The percentage adequacy both of overall oxygen delivery and of cardiac output response was low in every case, whether or not oxygen consumption and cardiac output themselves appeared to be abnormal when compared with grouped normal values. In other words when oxygen delivery and cardiac output at any one moment were viewed in relation to the needs of the subject's tissues at the same moment, a much sharper delineation of "adequacy" could be made than if they were compared to the responses of other subjects at other times. This approach to the adequacy of oxygen delivery and cardiac output responses has likewise been directed at the stages of "incipient" congestive heart failure. The present communication presents the results obtained in asymptomatic human subjects who exhibited clinical evidence of heart disease (Class I) and thus had clinical manifestations which are thought to represent earlier stages of a progression which leads ultimately to heart failure.

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[2] Work done in part during tenure of Research Fellowship and Fellowship as Established Investigator of the American Heart Association.

METHODS

Measurements of rate of oxygen consumption ($\dot{V}_{O_2}$), cardiac output ($\dot{Q}a$), arterial blood lactate and pyruvate concentrations per liter of blood water and total body water content (W_b) were carried out in the manner previously employed (1). The mild exercise (supine straight leg raising) was the same as that used for the study of normal subjects and of patients with congestive heart failure and approximated in severity the common daily activity of patients. It has been shown previously that the amount of exercise undertaken (in the "normal" range) has no effect on estimates of circulatory adequacy of the sort reported here (1). The 30 patients were asymptomatic and had never had dyspnea at any time. The diagnosis of heart disease was incidental to unrelated complaints at the time of the first examination. The lesions were chiefly valvular. In Group A, seven of the patients had mitral stenosis, two had predominantly mitral regurgitation, two had both stenosis and regurgitation without clear-cut predominance, two had tricuspid regurgitation, and two had "arteriosclerotic" (senile) heart disease. In Group B, six of the patients had aortic valvular deformity (two had stenosis and four had regurgitation or both stenosis and regurgitation), seven had moderately severe or severe essential hypertension, and two had pulmonic valvular stenosis. No edema or hepatomegaly was found on physical examination, and venous pressures, arm to tongue circulation times and vital capacities were normal. End diastolic pressures in the right ventricle were normal. All the patients considered themselves to be completely well and were either employed at full time jobs or were in school and active in sports. Thus the more numerous group who chose to restrict activity (as a result of medical advice or otherwise) are not represented because of the possibility of error in classification.

The derived quantities used in the section on results were calculated as follows from the observed data (1):

1) Excess lactate (XL) concentration:

$$XL = (L_n - L_0) - (P_n - P_0)(L_0/P_0).$$

L_n and L_0 are experimental (exercise) and control values of blood lactate, respectively; P_n and P_0 are the same for blood pyruvate.

2) Anaerobic metabolic rate (AMR):

$$AMR = dXL/dt \times 11.2\, W_b$$

given in ml. per minute of oxygen equivalents.

3) Percentage response of oxygen consumption (% resp. $\dot{V}_{O_2}$):

$$\% \text{ resp. } \dot{V}_{O_2} = \frac{\Delta\dot{V}_{O_2} \times 100}{\Delta\dot{V}_{O_2} + \text{AMR}}.$$

$\Delta\dot{V}_{O_2}$ is the change in $\dot{V}_{O_2}$ between the near-basal state and a steady state of exercise.

4) Percentage response of cardiac output (% resp. $\dot{Q}a$) and of arterial mixed venous blood oxygen difference [% resp. $(a - \bar{v})_{O_2}$]:

$$\% \text{ resp. } \dot{Q}a = \frac{\Delta\dot{Q}a \times 100}{\Delta\dot{Q}a + \dfrac{\text{AMR}}{(Ca_{O_2} - C\bar{v}_{O_2})_{\text{exercise (ex.)}}}}$$

$$\% \text{ resp. } (a - \bar{v})_{O_2} = \frac{\Delta(Ca_{O_2} - C\bar{v}_{O_2}) \times 100}{\Delta(Ca_{O_2} - C\bar{v}_{O_2}) + \dfrac{\text{AMR}}{\dot{Q}a_{ex.}}}.$$

$\Delta\dot{Q}a$ and $\Delta(Ca_{O_2} - C\bar{v}_{O_2})$ represent changes of cardiac output and arteriovenous oxygen difference between the basal state and the exercise; $(Ca_{O_2} - C\bar{v}_{O_2})_{ex.}$ and $\dot{Q}a_{ex.}$ represent the absolute arteriovenous oxygen difference and cardiac output in the steady state of exercise.

RESULTS

During the performance of mild exercise the 30 patients with heart disease increased oxygen consumption, cardiac output and arterial-mixed venous blood oxygen difference as did subjects without evidence of heart disease. The mean values at the end of exercise were: for $\dot{V}_{O_2}$, 456 ml. per minute per M.^{2} (standard deviation [σ] equals $\pm$78.9); for $\dot{Q}a$, 5.20 L. per minute per M.^{2} ($\sigma = \pm$1.49); and for $(Ca_{O_2} - C\bar{v}_{O_2})$, 70.8 ml. per L. ($\sigma = \pm$14.7). These levels represent changes from control of 282 ml. per minute per M.^{2}, 1.98 L. per minute per M.^{2} and 26.4 ml. per L. Average values found in normal subjects (1) were 433 ml. per minute per M.^{2} ($\sigma = \pm$81.2), 5.04 L. per minute per M.^{2} ($\sigma = \pm$1.35) and 71.3 ml. per L. ($\sigma = \pm$15.4), respectively. Statistically the heart disease group was not different from normal in these respects, and no single value fell outside the normal range. The two groups were likewise identical at rest with respect to these measurements: 154 ($\sigma = \pm$10.1) and 151 ($\sigma = \pm$9.4) ml. per minute per M.^{2} for $\dot{V}_{O_2}$, 3.22 ($\sigma = \pm$0.22) and 3.19 ($\sigma = \pm$0.29) L. per minute per M.^{2} for $\dot{Q}a$, and 44.4 ($\sigma = \pm$6.2) and 44.1 ($\sigma = \pm$5.1) ml. per L. for $(Ca_{O_2} - C\bar{v}_{O_2})$. The average increase in blood lactate during exercise was about the same in patients with heart disease as in normal subjects: 1.54 mM per L. ($\sigma = \pm$0.76) by comparison with 1.31 ($\sigma = \pm$0.60) mM per L. Changes in blood pyruvate also did not differ significantly from normal: 0.115 mM per L. ($\sigma = \pm$0.038) in the cardiac patients and 0.090 mM per L. ($\sigma = \pm$0.032) in normals. Some individual total lactate increases were very high in the patients, as shown in the diagram of these values in Figure 1. As previously pointed out (2), however, none of these data give any reliable indication whether oxygen supply to the tissues by way of the circulation was adequate or not, or whether the heart disease which was present had impaired cardiac performance.

Percentage response of oxygen consumption

From the relationships between the changes in lactate and pyruvate, calculations of anaerobic metabolic rate (AMR) were made as shown by Equations 1 and 2. The 30 patients with heart disease exhibited AMR values between 4.5 and 41.8 ml. oxygen equivalents per minute, averaging 32.3 ($\sigma = \pm$8.42), which are not significantly different from normal. The values of dXL/dt from which these are derived are shown diagrammatically in Figure 1. If these values are corrected for the varying severity of effort in different experiments by dividing by the total metabolic rates, the resulting indices average

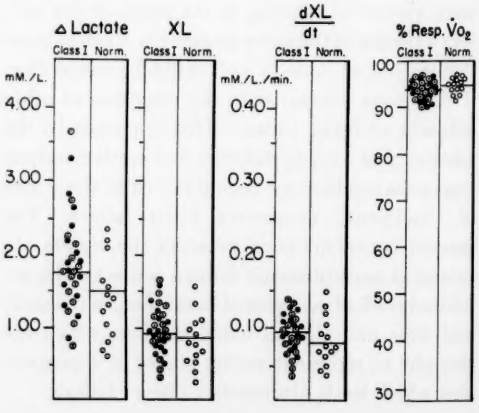

FIG. 1. CHANGES IN BLOOD LACTATE (Δ LACTATE), EXCESS LACTATE (XL), RATE OF ACCUMULATION OF EXCESS LACTATE (dXL/dt), AND PERCENTAGE RESPONSE OF OXYGEN CONSUMPTION IN SUBJECTS WITH CLASS I HEART DISEASE AND IN NORMAL SUBJECTS

In the Class I column, shaded points indicate Group A; open points, Group B.

0.059 ($\sigma = \pm 0.0185$), and are again not significantly different from normal. This figure may be otherwise expressed as the fractional adequacy of overall oxygen delivery or "percentage response of oxygen consumption" (% resp. $\dot{V}_{O_2}$), which averages 94.1 per cent ($\sigma = \pm 1.85$ per cent), and is depicted in Figure 1. This value permits the conclusion that 94.1 per cent of the oxygen required by body tissues during mild exercise was supplied at the time of the need, and is indistinguishable from the response of normal subjects who have no heart disease. This observation seems consistent with the fact that these cardiac patients were active and well.

Percentage response of cardiac output

It nevertheless remained to be determined whether an inadequate cardiac response could have been associated with this adequate overall oxygen delivery. The response of cardiac output may also be expressed as a fraction of what it should be (the "percentage response of cardiac output"), calculated as shown in Equation 4. For this purpose the patients with Class I heart disease are initially considered under two headings: *a*) "mitral stenosis" and *b*) "aortic stenosis." Certain other forms of heart disease are subsequently added to one or the other of these groups.

The percentage response of cardiac output (% resp. $\dot{Q}a$) was abnormal in all nine patients of Group A with atrio-ventricular valvular deformity. These figures are plotted in Figure 2 as scatter diagrams showing the % resp. $\dot{V}_{O_2}$ in the left hand column, % resp. $\dot{Q}a$ in the middle column and % resp. $(a - \bar{v})_{O_2}$ on the right. The three values obtained in a given subject in one exercise period are connected by lines to show their relationships. The range of values outlined by the block in the upper section of the chart encompasses the observations which have been made on normal subjects (1). The range blocked off in the lower section of the figure includes all values previously obtained in patients with definite congestive heart failure. All patients with recognizable mitral stenosis studied thus far have differed significantly, as shown in Figure 2, from both the normal and the heart failure groups in such a way that any single individual could have been placed properly with respect to diagnosis. These Class I patients invariably differed from those with mild heart failure in that they showed normal values for % resp. $\dot{V}_{O_2}$ and % resp. $(a - \bar{v})_{O_2}$ (above 90 per cent). However, each one differed from normal in having a reduced % resp. $\dot{Q}a$. This was also true of two patients with cardiac dilatation without valvular disease ("arteriosclerotic" or senile myocardial disease), and the results of these two studies are included in Figure 2. Although the clinical diagnosis of mitral regurgitation or tricuspid regurgitation is less certain than that of mitral stenosis in some asymptomatic subjects, the same findings were obtained in four such patients, and these are also included in Figure 2. Of the nine patients with apical diastolic murmurs, six were found to have no abnormality of pulmonary arterial or wedge pressure at rest or during exercise. Three patients showed some elevation of pulmonary wedge pressure (above 8 mm. Hg at rest; about 14 mm. in exercise), and these three had the most marked depressions of % resp. $\dot{Q}a$. Resting pulmonary

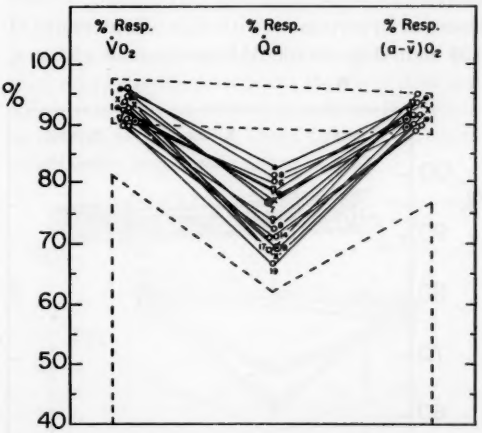

FIG. 2. PERCENTAGE RESPONSES OF OXYGEN CONSUMPTION, CARDIAC OUTPUT AND ARTERIOVENOUS OXYGEN DIFFERENCE IN PATIENTS OF GROUP A

Group A: Class I mitral stenosis (open circles), mitral regurgitation (closed circles), senile heart disease (crosses) and tricuspid regurgitation (triangles). The three values obtained for each individual are connected by lines. The total range of normal values (1) is shown by the upper box, that for patients with clinical heart failure by the lower box. Numerals beside points in the second column indicate the pulmonary wedge pressures in the patients with mitral stenosis. The ages of the patients, in order of decreasing % resp. $\dot{Q}a$, are as follows: 18, 27, 19, 24, 31, 20, 28, 33, 29, 19, 62, 21, 27, 57 and 30 years.

wedge pressures in patients with mitral stenosis are indicated in Figure 2 beside the points representing % resp. $\dot{Q}a$. Pressures were normal in the remainder of the 15 subjects.

Figure 3 shows the values of the three percentage responses in the 15 patients of Group B, with acceptable clinical evidence of semilunar valvular deformity or lesions having a similar hemodynamic effect on the heart. All asymptomatic patients with aortic stenosis who were studied presented findings which were indistinguishable from normal, as shown in Figure 3. This was also true of all patients studied who had systemic arterial hypertension and of two patients with pulmonic valvular disease. The findings were the same even when extreme cardiomegaly, electrocardiographic evidence of ventricular hypertrophy or angina pectoris were present. (In the cases with angina pectoris, of course, the term asymptomatic is applicable only in the sense that no exertional or nocturnal dyspnea and no fatigue or edema were noted.) Severe essential hypertension with diastolic pressures of 130 mm. Hg, Grade III eye ground changes, cardiomegaly and electrocardiographic changes likewise did not apparently alter the adequacy of cardiac output or of general oxygen delivery relative to peripheral tissue requirements.

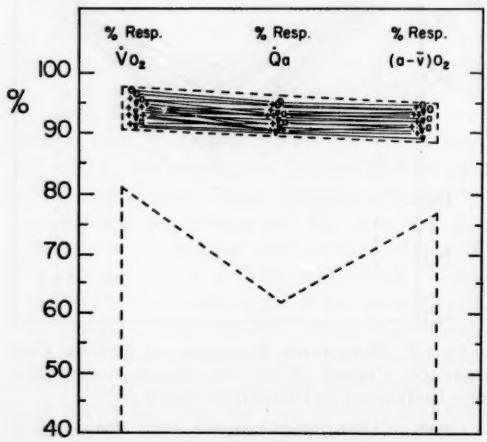

FIG. 3. PERCENTAGE RESPONSES OF OXYGEN CONSUMPTION, CARDIAC OUTPUT AND ARTERIOVENOUS OXYGEN DIFFERENCE FOR PATIENTS OF GROUP B

Group B: Class I aortic valvular disease (ovals, stenosis alone; triangles, regurgitation or combination), pulmonic valvular stenosis (squares) and systemic arterial hypertension (crosses). Ages of the patients, in order of decreasing % resp. $\dot{Q}a$, are as follows: 46, 48, 52, 39, 61, 54, 41, 59, 63, 45, 47, 56, 58, 49 and 50 years.

Effects of surgical treatment

Figure 4 depicts the findings in a small number of patients with mitral stenosis at varying intervals after apparently satisfactory surgical mitral commissurotomy. These studies were carried out because of the possibility that such patients may have been converted by therapy into "Class I" patients (in the objective sense which the present data suggest for this term). Although many such patients may not be classified truly in Class I (asymptomatic), the observations of Figure 4 are shown together because the varied results seem as unrelated to subjective symptoms as are the data obtained in the truly asymptomatic subjects previously described. The only striking improvement immediately after operation is shown in Figure 4, Part B, data from a 35 year old woman who was apparently greatly improved subjectively, although not completely well. As can be seen from the points at the top of the chart, two patients (two and three years postoperative) finally presented the percentage response findings expected in Class I mitral deformity and another (one and one-half years postoperative) approached this state.

Diagnostic evaluation of apical systolic murmurs

Figure 5 shows the data obtained thus far in a general survey of asymptomatic patients with apical systolic murmurs. Some of these subjects have the definite abnormality of % resp. $\dot{Q}a$ characteristic of atrio-ventricular valvular disease, while others show normal values. It has not appeared possible by clinical examination and use of familiar criteria (3) to predict these results in every case. Similar surveys of the physiologic significance of other early signs occurring alone (*i.e.*, opening snap, increased intensity of the first heart sound, and so forth) may prove to be of interest.

DISCUSSION

Occasional observations have been made in the past on patients with asymptomatic heart disease. The relationship of O_2-debt development

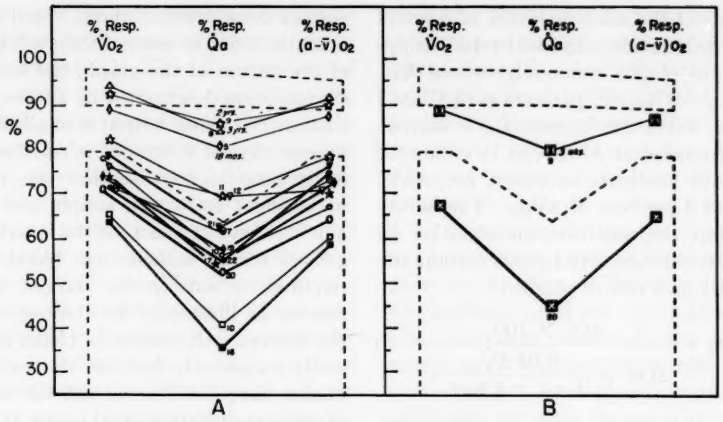

FIG. 4. EFFECT OF MITRAL VALVULOPLASTY (OPEN SYMBOLS) ON THE PERCENTAGE RESPONSES OF PATIENTS WITH MITRAL STENOSIS WHO HAD CLINICAL CONGESTIVE FAILURE PRIOR TO OPERATION (CLOSED SYMBOLS)

Section A shows lack of immediate effect in several patients (2 to 3 weeks postoperative except where otherwise indicated) with significant improvement in longer periods. Section B shows data from one patient (♀, Class II–III) who exhibited marked improvement immediately.

to severity of exercise has been found to be the same as in patients with no organic heart disease (4), but this relationship was also normal in various published data on congestive heart failure (1) and therefore offers little help in understanding the course of the disease. Resting and exertional lactate concentrations in arm venous blood have been noted to be normal in several such patients (5). Richards (6) stated that no circulatory abnormalities were demonstrable on cardiac catheterization. However, reduced renal blood flow has been found in asymptomatic patients with heart disease (7).

Although the present observations indicate that high levels of total lactate may occur in the arterial blood of a small proportion of patients with asymptomatic heart disease, most of these patients are indistinguishable from normal persons in this respect. Similar unusual degrees of lactate accumulation may often be seen in normal subjects who believe they have heart disease and feel anxiety about physical exertion; this finding seems more probably related to the psychogenic ventilatory response to exercise (8). In any event, the rate of accumulation of "excess lactate" or the rate of tissue anaerobic metabolism in mild exercise is only about 6 per cent of the total energy requirement and therefore does not differ from that of normal people as far as can be determined, even in those patients in whom total lactate response is exaggerated.

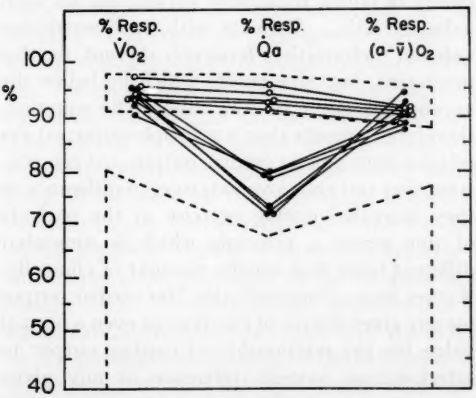

FIG. 5. PERCENTAGE RESPONSES IN A GROUP OF ASYMPTOMATIC SUBJECTS WITH NO OTHER CLINICAL ABNORMALITY THAN AN APICAL SYSTOLIC MURMUR

The data tend to fall into two groups, one of which is normal (open circles, falling inside the upper block denoting normal range), the other definitely abnormal by the criteria of Figure 2.

On the basis of the small variance of some of the data shown, certain empirical relationships may be said to obtain commonly among $\dot{V}_{O_2}$, $\dot{Q}a$, and $(Ca_{O_2} - C\bar{v}_{O_2})$ in patients with Class I heart disease. Since the % resp. $\dot{V}_{O_2}$ is normal, it may be assumed that AMR can be estimated from $\Delta\dot{V}_{O_2}$ with moderate accuracy, *i.e.*, AMR averages about 5 per cent of $\Delta\dot{V}_{O_2}$. The following relationship, derived from the equation for % resp. $\dot{Q}a$, would therefore have a certain statistical validity as a rule of thumb:

$$\%\ \text{resp.}\ \dot{Q}a = \frac{\Delta\dot{Q}a \times 100}{\Delta\dot{Q}a + \dfrac{0.05\,\Delta\dot{V}_{O_2}}{(Ca_{O_2} - C\bar{v}_{O_2})_{ex.}}}. \quad 5)$$

This empirical equation is derived from the data obtained from the present normal subjects and patients with asymptomatic aortic or pulmonic valvular disease or essential hypertension. In normal subjects % resp. $\dot{Q}a$ is also an empirical constant, and the equation therefore simplifies to a more basic principle:

$$\Delta\dot{Q}a = K\frac{\Delta\dot{V}_{O_2}}{(Ca_{O_2} - C\bar{v}_{O_2})_{ex.}}. \quad 6)$$

The mean value of the constant K is 0.625.[3] If this equation is plotted as cardiac output against arteriovenous oxygen difference, the result is a family of falling hyperbolic curves, one for each value of $\Delta\dot{V}_{O_2}$. Patients with atrioventricular valvular deformities, however, do not fit this prediction, but give points which lie below the appropriate curve in each case. The equation, therefore, suggests that a principle governed the relationships among cardiac output, oxygen consumption and arteriovenous oxygen difference as they increased during exercise in the patients of this series, a principle which is somewhat different from that usually thought of clinically. Rather than a "normal value" for cardiac output for any given degree of exertion, or even a normal value for the relationship of cardiac output to arteriovenous oxygen difference at any given oxygen consumption, there would appear to be a whole series of normal values lying along one of the curves of the graph, the locations of the curves being determined by the value of K. If a patient's cardiac output is small and his arteriovenous oxygen difference is relatively large, one is tempted to suppose that this represents an impairment to oxygen supply and therefore an abnormality. In fact, as the arteriovenous oxygen difference widens, less blood flow will be required to supply the oxygen needs of the tissues, as illustrated by the negative slopes of the curves. Of course a blood flow which is really excessively low for the existing arteriovenous oxygen difference will fall below all parts of the appropriate normal curve, but the finding *per se* of a larger arteriovenous difference and smaller cardiac output in one group of subjects than another does not mean that cardiac output is "insufficient."

Application of Equation 5 to 137 published studies of normal cardiac output by various methods in exercise gives estimated percentage responses of cardiac output of 89 per cent or more (9–17). Data from one patient with Class I mitral disease have been given previously (18) and show a significantly reduced % resp. $\dot{Q}a$, while two patients with Class I aortic valvular disease (19) showed normal values.

The finding of definite abnormalities of cardiac output response in the early stages of the development of chronic mitral stenosis suggests the possibility that a more prolonged course of events than is usually supposed may form the physiologic history of congestive heart failure of this etiology. Judging from the abnormalities in these Class I patients (*i.e.*, those with early disease), the years during which the obvious dyspnea of mitral stenosis appears and grows worse may be only the last third of the course of continuous circulatory change which, like the endocarditis itself, began long before the first dyspnea appeared.

Several questions are raised by this interpretation. The first is in regard to the possible mechanism of a limitation of cardiac output response apparently produced by such early deformity of the valve. Lewis (20) held that blood flow could not be importantly affected until the valve orifice had been markedly reduced, and

[3] While this equation may at first seem to resemble that of the Fick principle (which, of course, expresses all possible values without selection), it is well to point out that the Fick principle dictates only the following relationship among the terms of Equation 6:

$$\Delta\dot{Q}a = \frac{\dot{V}_{O_2\,ex.}(Ca_{O_2} - C\bar{v}_{O_2}) - \dot{V}_{O_2}(Ca_{O_2} - C\bar{v}_{O_2})_{ex.}}{(Ca_{O_2} - C\bar{v}_{O_2})(Ca_{O_2} - C\bar{v}_{O_2})_{ex.}}.$$

this has also been the interpretation placed on results of certain experiments on hydraulic systems and on animals (21–23). Explanations of the present results presumably must lie in the slight interference to blood flow through the valve produced by turbulence in the stream, but Class I aortic valvular deformity does not produce the same result. The pressure available for effecting flow through atrioventricular valves is low at this stage. Compensation by the pulmonary venous bed for a slight resistance to flow cannot occur in the same way as if blood were driven through the valve directly by ventricular contraction. An adequate increase in pressure at this time would presumably restore blood flow to normal. If it can be supposed that the pressure gradient required to overcome the early valvular deformity is eventually provided, by the time the pressure has risen the valvular deformity must also have progressed since the percentage response of cardiac output was not found to be improved in the subjects with elevated pulmonary wedge pressures, but was either the same or lower.

A second question is in regard to the lowered oxygen consumption response, which was found only in patients in whom the adequacy of cardiac output was below about 70 to 75 per cent [the lowest seen in the present data, all lower values having been found in patients with heart failure and reduced % resp. $\dot{V}_{O_2}$ (1)]. Whether the ultimate appearance of insufficiency of overall oxygen supply may be related causally to the appearance of clinical heart failure, which seems to occur only in its presence, is not evident from the available data, but is suggested by the fact that no cases have yet been observed which fall into an intermediate zone, *i.e.*, patients who show a reduced % resp. $\dot{V}_{O_2}$ with no symptoms or signs of cardiac failure, or who show clinical heart failure without reduction of % resp. $\dot{V}_{O_2}$. Continued study may reveal that there are such cases, however.

No such physiologic history preceding the heart failure of aortic valvular deformity or systemic hypertension appears to be implied by the present observations on the early stages of these lesions. It is possible that the adequacy of cardiac output remains unimpaired until clinical heart failure has actually occurred. After this event the physiologic findings have been shown to be the same, whatever the etiology of the heart failure (1). An explanation for this difference may perhaps be found in the fact that blood is propelled through the semilunar valves by the immediately proximal muscular ventricle, which is known to increase its force of contraction in response to increased resistance to flow (24, 25). If the present patients are representative of the group as a whole, more or less complete compensation for the valvular deformity by the ventricle may continue until the ventricular muscle itself fails. Corollary events in the life of such a patient might be expected, therefore, to be somewhat different from those produced by atrioventricular valvular disease with its continuous course, physiologically speaking, almost from the first.

In patients with aortic valvular disease there thus appears to be some physiologic basis for understanding that 40 years of active, normal life elapse after acute rheumatic fever, only to be ended by a period of congestive heart failure which runs its course to a fatal termination in a few months (26). This pattern is quite different from one of 10 years of health followed by 5 or more years of symptomatic cardiac insufficiency, the course described for atrioventricular valvulitis by the statistics of DeGraff and Lingg (27), even though their patients were selected for severity of inflammatory disease (by virtue of giving a definite history of acute rheumatic fever) and the mean time of death was not corrected for causes other than cardiac decompensation. The present findings might also be taken to suggest that semilunar valvular deformity would have no influence on the physiologic history of atrioventricular valvular disease if the two occurred together; clinical observations indicate a similar principle may apply to the symptomatic history (28). Students of the clinical course of aortic valvular disease have repeatedly remarked on its prolonged and benign behavior prior to decompensation, and the rapid demise afterward (29–31). These observations are a reflection on the occurrence of congestive heart failure and not on the unpredictable possibilities of sudden death or bacterial endocarditis. The generalizations, of course, are subject to individual variations. Experience has shown that untreated systemic

arterial hypertension likewise produces no limitation of activity over a major portion of the patient's lifetime, although many nervous symptoms may be present; heart failure may occur rapidly, however, and in severe hypertension progress precipitously.

In view of the supposed implications of the present results with regard to the adequacy of the responses of the heart to meet body needs, it is interesting that the age of the patients also seemed to have no effect on the findings. The difficulty of subdividing the narrowly-grouped figures in the normal range, however, has already been mentioned. There may be some broad tendency toward age dependence in all the groups, but the statistical correlation is not significant. A 3 to 4 per cent variation of the percentage responses in the normal group and among the patients with aortic and pulmonic valvular disease and essential hypertension can only be regarded as unexpectedly constant, even assuming a single population of data.

The course of change of the findings in the present small group of patients during two to three years following mitral valvuloplasty might be interpreted as being the reverse of the physiologic pathogenesis of mitral stenosis. The almost complete lack of change in percentage response of cardiac output two to three weeks after surgery in most patients is difficult to explain, since pulmonary arterial and "wedge" pressures were reduced. Mitral regurgitation may replace mitral stenosis in some of these patients, and the characteristic low pulmonary vascular pressures associated with mitral regurgitation may be present; adequacy of cardiac output responses, on the other hand, may not always be improved by this change.

The findings in two cases of unexplained cardiac dilatation may possibly serve to indicate that the course of development of heart failure from senile or "arteriosclerotic" myocardial disease is more nearly like that of Group A (atrioventricular valvular disease) than Group B. It may, however, be shorter in duration than that of mitral stenosis, judging from the subsequent history of these patients. Both patients subsequently developed frank congestive heart failure, one within a year, the other after 26 months, but were easily controlled with digitalis. All but four of the remaining subjects of Group A have remained well after one to four years. Four patients with mitral stenosis (including the three with elevated pulmonary vascular pressures) began to suffer slight dyspnea on exertion two to four years after the study, and the dyspnea grew worse over a period of six months or more. Two patients of Group B (aortic stenosis and regurgitation) subsequently died of rapidly progressive congestive heart failure after remaining well for 16 and 34 months, respectively, following the study. It is interesting to speculate that the Group A findings (depression of % resp. $\dot{Q}a$) will in the future probably be found in some hypertensive patients when a larger number have been studied, since the development of senile or "arteriosclerotic" heart disease is assumed to account for the ultimate occurrence of heart failure in at least some patients with essential hypertension. The same speculation may also be entertained about "normal" subjects as the number of observations increases, since unrecognized cardiac dilatation or "aphonic" mitral stenosis might occur among presumed normal subjects.

Some of the physiologic characteristics of the calculated "percentage response of cardiac output" have been given previously, and these may be extended somewhat on the basis of the present data. This value may have any magnitude equal to or less than % resp. $\dot{V}_{O_2}$. It now appears possible to state empirically that if % resp. $\dot{Q}a$ does have a value less than % resp. $\dot{V}_{O_2}$, heart disease will be found to be present, even if % resp. $\dot{V}_{O_2}$ is normal. Of course if % resp. $\dot{V}_{O_2}$ were 95 per cent, one would conclude that the energy requirement of the tissues only slightly exceeded the total oxygen transport, although the requirements might at the same time exceed the blood flow response considerably. Aside from this tentative empirical conclusion on the association of heart disease and reduced % resp. $\dot{Q}a$, based on the present observations, the theoretical basis of this principle may be deduced from the Fick equation $(Ca_{O_2} - C\bar{v}_{O_2} = \dot{V}_{O_2}/\dot{Q}a)$ which indicates the rise in $(Ca_{O_2} - C\bar{v}_{O_2})$ which will occur if increase in oxygen utilization by tissues occurs at a greater rate (percentage-wise) than the simultaneous increase in blood flow, even without considering the possibility of an AMR in addition. This phenomenon, of course,

is generally recognized. From the equations for percentage response of cardiac output and percentage response of oxygen consumption, it is apparent that for any value of % resp. $\dot{V}_{O_2}$ (*i.e.*, for any given value of the relationship between anaerobic metabolic rate and change in oxygen consumption) % resp. $\dot{Q}a$ can be less than this in only one circumstance, *i.e.*, if the cardiac output has failed to increase in some "proper" proportion to oxygen utilization. This conclusion, however, must be regarded as purely qualitative. *How much* less the % resp. $\dot{Q}a$ will be is another question. From the equation, one may say that this will not be determined by the size of the arteriovenous oxygen difference, but by the relationship between arteriovenous oxygen difference and the magnitude of the anaerobic metabolic rate. The selective depression of % resp. $\dot{Q}a$ below % resp. $\dot{V}_{O_2}$, therefore, apparently always represents the effect of reduced relative cardiac response whatever may be the values of other parameters, including % resp. $\dot{V}_{O_2}$ or $\dot{Q}a$ itself, or the presumed clinical condition of the patient.

When observations of the sort presented here are made for diagnostic purposes in patients of unknown cardiac status, great care is used in establishing an initial "near-basal" state. However, no attempt is made to regulate the amount of exercise performed or its duration beyond about three to four minutes, which appear to be required for stability of $\dot{V}_{O_2}$. Moreover, examination of the figures for total body water reveal that this determination may be omitted in subjects judged to be of "normal" weight and build without introducing sufficient error in the calculation of percentage responses to affect the general clinical conclusions [W_b being estimated from body weight (31)]. Asymptomatic subjects with apical systolic murmurs have been briefly surveyed, as shown in Figure 5. While a final judgment of the medical significance of some of the abnormal findings will require a long term follow-up, it is noteworthy that a portion of this preliminary group of patients show percentage responses which are abnormal in the same way that patients with definite mitral stenosis are abnormal. Among the six patients who thus gave physiologic evidence of atrioventricular valve abnormalities, two were evaluated clinically as having normal hearts. Among the five patients with percentage response values indistinguishable from normal, two had been diagnosed clinically as having mitral valvular disease, at least tentatively. The data cannot indicate whether semilunar valvular abnormalities might have been present in any of the patients, but congenital septal defects probably may be excluded in these subjects because cardiac catheterization failed to give any evidence of them, and because certain characteristic effects on percentage responses produced by such lesions were not present; these effects cannot be adequately described here and will be presented elsewhere.

None of the data reported here or previously (1) include any observations made on subjects whose clinical state was impossible to evaluate, or at least categorize, by independent means. Needless to say, this is a most important group of patients. Thus far, however, the findings obtained by the present methods in such patients have fallen distinctly into one or another of the categories described; they have often been perfectly normal, but on occasions have shown the percentage responses characteristic of Class I heart disease or even of congestive heart failure (1).

SUMMARY AND CONCLUSIONS

1. Patients with clinical evidence of heart disease but no symptoms of congestive heart failure have an adequate response of the oxygen transport system during mild exercise so that body tissues are forced to rely on anaerobic metabolism to only a very small extent.

2. Patients with atrioventricular valvular deformities may have an inadequate response of cardiac output before any other measurable physiologic change occurs, and this may appear and grow worse for at least four years before clinical cardiac insufficiency appears.

3. By contrast, patients who have had semilunar valvular deformities or essential hypertension for years, even with evidence of myocardial deterioration, usually have an adequate cardiac output response unless full blown congestive heart failure is present.

4. Changes in cardiac output and arteriovenous oxygen difference are not reliable indications in themselves of normal or adequate cardiac responses.

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STUDIES OF THYROID FUNCTION AND THE PERIPHERAL METABOLISM OF I^{131}-LABELED THYROXINE IN PATIENTS WITH TREATED GRAVES' DISEASE [1, 2]

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Recent studies have revealed an abnormality in the peripheral metabolism of thyroxine in patients with active untreated Graves' disease (1). In such patients, an abnormally large fraction of the extrathyroidal pool of thyroxine is degraded daily.

Other authors have also observed an accelerated fractional turnover of thyroxine in patients with untreated Graves' disease (2, 3) and have postulated that the rate of turnover of the hormone might be a function of its concentration in the blood (2). Alternatively, accelerated turnover of thyroxine might merely reflect the general speeding of metabolic processes which occurs during the hyperthyroid state. The present studies were designed to differentiate between these and other possibilities. Rates of turnover of thyroxine have been assessed in patients in whom the thyrotoxicity had been corrected by appropriate therapy, as well as in nonthyrotoxic subjects who were made hypermetabolic by the administration of thyroid hormone. A preliminary report of the data obtained has been published in abstract form (4). It has been shown that the induction of thyrotoxicosis medicamentosa does result in an augmentation of the fractional rate of turnover of thyroxine. However, it has also been demonstrated that accelerated fractional turnover of thyroxine frequently persists in patients with Graves' disease, despite prolonged correction of the hypermetabolic state, and despite reduction of the protein-bound iodine (PBI) to normal or subnormal values. It is apparent, therefore, that the increase in the fractional rate of thyroxine turnover which occurs in patients with Graves' disease cannot be entirely ascribed to either the concentration of circulating thyroid hormone or the immediate metabolic status of the patient.

PATIENT MATERIAL

(Table I). Forty determinations of the rate of turnover of thyroxine were made in 23 patients with treated Graves' disease. With but 7 exceptions, all patients had been followed continuously by the authors since the diagnosis of Graves' disease had first been made.

Twenty-two tests were performed in 15 patients who were being treated with propylthiouracil or methylmercaptoimidazole. Duration of symptomatic remission ranged from 2 to 26 months and averaged 12 months. In four instances, tests were performed following 1 month in which antithyroid medication was supplemented by the administration of Lugol's solution, 10 drops, three times daily. Seven additional tests were performed in six of these subjects from 4 to 14 months following withdrawal of propylthiouracil. In these subjects, symptomatic remission had been effected for 17 to 39 months.

Nine tests were performed in seven subjects who had been treated by subtotal thyroidectomy from 7 months to 10 years earlier. One of these patients (No. 12) had previously been studied while receiving antithyroid therapy.

Measurements of the rate of turnover of thyroxine were also made in one patient treated with Lugol's solution and another patient treated with radioactive iodine, five and six years earlier, respectively.

Values were compared with those obtained in normal subjects and in patients with untreated myxedema or thyrotoxicosis. Previously reported data for subjects in these categories (1) have been supplemented by the study of additional patients.

In order to ascertain the reproducibility of the measurement, two determinations of thyroxine turnover were secured in six normal subjects at intervals of three to

[1] This investigation was supported in part by Research Grant No. A-267 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, and in part by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-412.

[2] Presented in part at the 1955 meeting of the American Society for Clinical Investigation, May 2, 1955, Atlantic City, N. J.

eight months. Values of thyroxine half-time did not vary by more than 0.3 day.

Methods

Thyroxine turnover. The distribution and rate of turnover of thyroxine were measured by a method described previously (1). Briefly, thyroxine (4 to 5 μg.), labeled with 30 μc. of I^{131} [3] was injected intravenously and total daily urinary collections as well as daily specimens of plasma were obtained for the ensuing 9 to 12 days. The virtual volume of distribution of thyroxine was calculated daily. After complete equilibration of the injected isotope, as evidenced by constancy of its volume of distribution, the thyroxine half-time was estimated from the slope of the curve depicting the concentration of the radioactivity in the serum. The fractional rate of turnover of thyroxine was calculated as the quotient, 0.693/half-time (day). Daily degradation of hormonal iodine (daily degradation rate) was calculated as the product, PBI (μg. per L.) × volume of distribution (liters) × fractional turnover rate (per cent per day).

In several instances, in order to avoid economic hardship to the patient, studies were performed on an outpatient basis. In such cases, urinary collections were not made. Fractional rates of turnover of thyroxine were estimated from the slope of the time-concentration curve of radiothyroxine in plasma, employing only data obtained subsequent to the first 48 hours following injection of the tracer. The volume of distribution of thyroxine was estimated, using the isotope dilution principle, by extrapolation of this portion of the curve to zero-time (the time of injection).

It had previously been noted, in patients with untreated Graves' disease, that accelerated fractional turnover of thyroxine rapidly liberated radioiodide which was quickly accumulated by the thyroid gland and released as labeled hormone. This recycled moiety produced an apparent retardation of the rate of disappearance from the plasma of the initially injected radiothyroxine (1). This difficulty could be circumvented if thyroidal reutilization of liberated radioiodide were completely inhibited by antithyroid drugs. Accordingly, all patients in the present studies were given 30 mg. of methylmercaptoimidazole every six hours during the testing procedure. By direct counting over the neck, this dosage of drug was shown to prevent appreciable thyroidal accumulation of the radioiodine liberated by the degradation of labeled thyroxine.

In all instances, determination of the 24 hour thyroidal percentage accumulation of a tracer dose of radioiodine as well as measurements of basal metabolic rate were made within the week prior to the determination of the thyroxine turnover rate. In patients receiving antithyroid drugs, medication was continued in its usual dosage during the measurement of thyroidal radioiodine uptake. Tracer doses of inorganic iodide were sufficiently small (3 μc.) so that any accumulated I^{131} released during the subsequent turnover study would not obscure the true rate of disappearance of injected radioactive thyroxine from the plasma.

Thyroxine-binding protein. In eight patients who displayed persistently accelerated turnover of thyroxine, the capacity of the serum's inter-alpha migrating thyroxine-binding protein (TBP) to bind thyroxine was assessed by techniques described in detail elsewhere (5). Sera from these and from normal control subjects were enriched with stable and with I^{131}-labeled thyroxine in amounts sufficient to achieve four standard concentrations of added thyroxine over the range from 10 to 160 μg. per cent. Sera were then subjected to electrophoresis in filter paper, using veronal buffer, pH 8.6, ionic strength 0.05. The distribution of radioactivity in the individual electrophoretograms was ascertained in an automatically recording strip-scanner. Scanning patterns were then traced onto hard paper of uniform thickness. The weight of the tracing of the radioactive components associated with albumin and with TBP were determined on an analytical balance, and were expressed as a percentage of the total weight of the tracing. Values from normal subjects and from patients with treated Graves' disease were compared.

Suppression of thyroidal uptake of I^{131}. On 30 occasions in 21 patients, the ability of exogenous thyroid hormone to suppress thyroidal accumulation of radioiodine was assessed. Three patients were studied before and after the oral administration of 75 μg. of triiodothyronine daily for three weeks. Eighteen patients received desiccated thyroid (U.S.P.) according to the following dosage schedule: following three weeks of an initial dosage of 2 or 3 gr. daily, thyroidal uptake was repeated. If suppression had not been achieved, the dosage was increased by 2 gr. daily during each two week period until suppression was achieved or until a dosage of 8 or 9 gr. daily had been employed. In one patient (No. 13), this regimen was discontinued at a dosage of 5 gr. daily because of severe symptoms of thyrotoxicity. Suppression was considered to have occurred if the 24 hour thyroidal uptake of radioiodine was decreased either to less than 10 per cent of the administered dose or to less than half of its initial value.

In six of the patients in whom suppression was achieved, thyroidal uptakes were allowed to return to control values. During the ensuing three to four weeks, patients were given quantities of inorganic iodine equal to the iodine content of the suppressive dose of desiccated thyroid. Thyroidal uptake was then re-evaluated.

Induction of thyrotoxicosis medicamentosa (Table II). The effect of artificially induced thyrotoxicosis on the peripheral turnover of thyroxine was assessed in four patients. One patient with nontoxic nodular goiter was given 10 gr. of desiccated thyroid daily. One patient with treated Graves' disease, one with nontoxic goiter, and one patient with primary amenorrhea were given 200 μg. of triiodothyronine daily. Studies of the peripheral turnover of thyroxine were performed before and at the end of a 60 day treatment period.

[3] Obtained from Abbott Laboratories, Oak Ridge, Tenn.

TABLE I

Summary of data obtained in patients with treated Graves' disease

Patient	Sex	Age	Rx	Duration of remission (*months*)	Thyroxine half-time (*days*)	Protein-bound iodine (*μg. %*)	Organic iodine degradation (*μg./day*)	Thyroidal 24-hour I^{131}-uptake (*% dose*)	Suppression	Basal metabolic rate
				Studied during antithyroid therapy						
1	F	33	PTU*	7	5.9	6.0	77	62	—	+ 3
			PTU	14	5.2	5.2	64	61	Yes	+ 7
2	F	34	PTU & Lugol's	11	5.5	—	—	—	—	− 7
			PTU	17	5.1	5.8	97	80	No	− 1
3	M	43	PTU	8	3.9	1.2	67	74	Yes	+ 3
			PTU & Lugol's	14	4.9	—	—	—	—	+ 5
4	F	35	PTU	5	4.8	2.2	26	55	Yes	+12
5	F	35	PTU	14	5.0	3.5	60	59	Yes	0
6	M	43	PTU	6	4.0	2.4	37	52	Yes	− 5
			PTU	22	4.6	4.0	50	46	Yes	+ 3
7	F	37	MMI†	18	5.2	1.5	22	92	Yes	−27
8	M	38	PTU	26	5.8	4.7	56	43	Yes	+ 4
9	F	47	PTU	2	5.2	5.7	59	56	No	+10
			PTU & Lugol's	4	5.8	—	—	—	—	+ 4
10	F	62	PTU	10	4.5	1.7	23	68	Yes	−15
			PTU & Thyroid	15	3.8	6.3	102	(2)‡	Yes	+18
			PTU & Lugol's	19	3.4	—	—	—	—	+ 6
11	M	15	PTU	6	4.0	3.6	46	89	Yes	+ 3
12	F	40	PTU	6	3.6	4.5	71	40	No	+ 7
13	F	48	PTU	10	6.0	4.4	46	70	No	+ 5
14	F	44	PTU	7	4.6	2.8	40	85	Yes	− 1
15	F	46	MMI	8	4.9	1.9	23	49	Yes	−30
			Mean	12	4.8	3.7	54	64		0
			Std. dev.	6	0.8	1.6	25	16		12
				Studied after cessation of antithyroid therapy						
1	F	34	PTU	27	5.2	6.5	85	45	—	+ 7
2	F	35	PTU	29	6.3	6.1	67	34	Yes	+ 8
4	F	36	PTU	17	5.5	5.6	62	70	Yes	+ 3
		37		35	7.9	6.2	50	58	Yes	− 1
5	F	37	PTU	39	5.4	5.2	74	53	—	+ 6
11	M	16	PTU	20	4.1	5.0	70	65	Yes	+10
14	F	45	PTU	20	5.8	5.4	68	47	Yes	− 2
			Mean	27	5.7	5.7	68	53		+ 4
			Std. dev.	8	1.2	0.5	11	12		5
				Studied after surgical therapy						
12	F	41	—	18	5.0	3.8	57	46	Yes	+ 4
		42	—	31	4.6	4.0	67	24	Yes	− 5
16	M	34	—	7	5.2	5.1	67	53	—	+ 2
		35		21	5.8	4.6	57	48	Yes	+ 1
17	F	36	—	10 yrs.	6.2	6.5	73	30	Yes	+ 5
18	F	65	—	5 yrs.	6.6	5.0	47	81	—	+12
19	F	67	—	4–1.5 yrs.§	7.1	6.9	63	—	—	− 4
20	F	53	—	10 yrs.	7.1	3.5	35	22	Yes	−10
21	F	21	—	7 yrs.	6.1	5.0	51	32	Yes	− 5
			Mean	4.7 yrs.	6.0	4.9	57	42		0
			Std. dev.	3.6	0.9	1.2	12	20		7
				Miscellaneous therapy						
22	F	24	Lugol's	5	4.8	5.0	43	18	Yes	+ 8
23	F	53	I^{131}	6	5.1	5.6	64	54	Yes	+ 6
			Grand mean	28	5.2	4.5	57	55		+ 1
			Std. dev.	29	1.0	1.4	19	19		9

* Propylthiouracil.
† Methyl-mercaptoimidazole.
‡ Value obtained during suppression with desiccated thyroid.
§ Surgery 1.5 years after initiation of antithyroid therapy.

TABLE II

The effect of diiodotyrosine and of induced hypermetabolism on the peripheral degradation of thyroxine

Patient	Diagnosis	Status	Thyroxine half-time	PBI	Thyroxine degradation	BMR
			days	*μg. %*	*μg. I/day*	
17	Treated Graves' disease	Control	6.2	6.6	74	+ 3
		Diiodotyrosine*	6.0	7.4	82	+ 6
24	Nontoxic goiter	Control	5.9	5.2	54	−10
		Diiodotyrosine*	6.0	5.3	55	−12
20	Treated Graves' disease	Control	7.1	4.8	42	+ 3
		Diiodotyrosine*	6.8	5.1	47	+ 5
		Triiodothyronine†	5.0	1.7	(21)§	+28
25	Nontoxic goiter	Control	8.0	6.8	58	−15
		Triiodothyronine†	5.1	1.7	(19)	+25
26	Secondary amenorrhea	Control	8.7	6.7	44	+ 5
		Triiodothyronine†	4.5	4.6	(32)§	+31
27	Nontoxic goiter	Control	6.8	6.0	70	+ 3
		Desiccated thyroid‡	5.2	12.3	170	+41

* Chromatographically pure diiodotyrosine, 500 μg. P.O. daily for 60 days.
† Cytomel®, 200 μg. P.O. daily for 60 days.
‡ Desiccated thyroid, U.S.P., 10 gr. daily for 60 days.
§ Values in patients receiving triiodothyronine, indicated in parentheses, are presumed to be inaccurate, since neither the rate of turnover of triiodothyronine nor its percentage contribution to the PBI were determined.

In addition, studies of thyroxine turnover were performed in two patients with treated Graves' disease and one patient with nontoxic nodular goiter before and after a 60 day treatment period with 500 μg. daily of diiodotyrosine.

Chemical analysis and radioactive assay. Concentrations of protein-bound iodine (PBI) were determined by the method of Barker, Humphrey and Soley in sera obtained from each patient at the beginning and the conclusion of the thyroxine turnover study (6).[4] Assays for radioactivity in blood, urine, and thyroid gland were performed as previously described (1).

RESULTS (TABLE I)

Patients studied during therapy with antithyroid drugs

In 22 tests performed in 15 patients, thyroxine half-time averaged 4.8 ± 0.8 days (mean ± standard deviation), a value significantly different from the normal of 6.8 ± 0.5 ($p < 0.001$). Only four subjects (Nos. 1, 8, 9, 13) exhibited half-times within the normal range (Figure 1). Duration of remission averaged 12 months in both these subjects and in the subjects with abnormally rapid turnover of thyroxine. Of the six patients who were studied more than once during antithyroid therapy, three demonstrated a decrease in half-time between successive tests, and three demonstrated an increase. Valid estimations of PBI could be obtained during 18 tests. These ranged between 1.2 and 6.3 μg. per cent and averaged 3.7 ± 1.6 (Figure 2). Values less than 3.0 μg. per cent were found in seven instances (mean, 2.0 μg. per cent), and three of these seven patients (Nos. 7, 10, 15) displayed enlarging goiter, subnormal basal metabolic rate (BMR), delayed relaxation of the deep tendon reflexes, and other stigmata of hypometabolism at the time of the measurements. Thyroxine half-times averaged 4.9 days in the three hypometabolic subjects and 4.3 days in the remaining four patients with subnormal PBI's.

Within the entire group, daily degradation of organic iodine averaged 54 ± 25 μg. This value is not significantly different from the normal mean (55 ± 12). Among the seven patients with subnormal PBI's the three hypometabolic individuals displayed degradation rates averaging 22 μg. of iodine per day. A fourth patient (No. 4) demonstrated no metabolic symptoms despite a daily degradation rate of 26 μg. of hormonal iodine. In the remaining three patients with low PBI's degradation ranged between 37 and 67 μg. of iodine

[4] Performed by Bio-Science Laboratories, 2231 South Carmelina Avenue, Los Angeles, Calif.

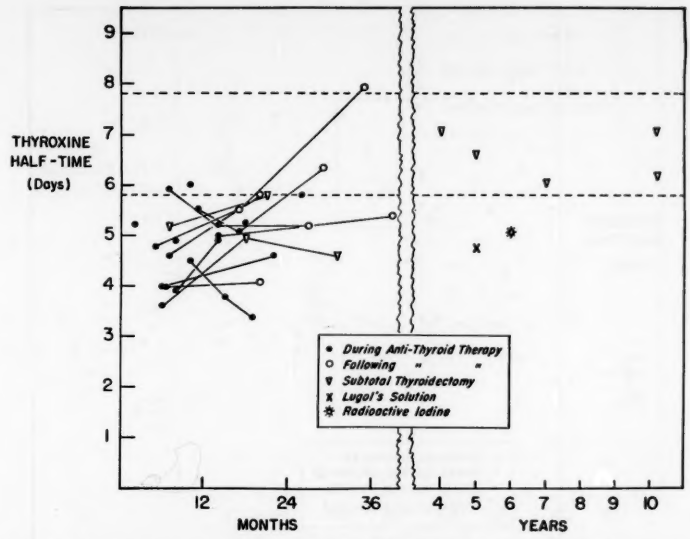

FIG. 1. THE RELATIONSHIP BETWEEN THE DURATION OF SYMPTOMATIC REMISSION AND THE RATE OF TURNOVER OF THYROXINE PERIPHERALLY

Dotted lines represent the normal mean thyroxine half-time ± 2 standard deviations, based on observations in 30 normal subjects of all ages.

per day, despite the reduction in the concentration of circulating thyroid hormone.

Values for the 24 hour thyroidal uptake of I^{131} averaged 64 ± 16 per cent for the entire group, and only four values of less than 50 per cent were noted. The latter value is considered to be the upper limit of normal in this laboratory. Thyroidal uptakes in the seven patients with subnormal PBI's averaged 68 per cent. In the four instances in which it was assessed, no demonstrable discharge of thyroidal radioactivity followed the oral administration of 1.0 Gm. of potassium thiocyanate.

Suppression of thyroidal uptake by exogenous hormone was assessed 17 times in 15 patients, and was achieved in 13 instances. In 3 of the 4 instances in which suppression was not achieved, subnormal values for thyroxine half-time were present. Except in 1 patient (No. 3) in whom 5 gr. was required, the daily dose of desiccated thyroid necessary to effectuate suppression ranged between 2 and 4 gr. In 6 patients, administration of inorganic iodine in doses equivalent to the iodine content of the suppressive dose of hormone failed to alter the thyroidal uptake of I^{131}.

Patients studied after completion of antithyroid therapy

Subnormal values of thyroxine half-time were found in four tests in four of six patients. In the other three tests, including one test performed in a patient whose previously determined half-time had been short (No. 4), normal values were seen. In four of the six patients, thyroxine half-time was longer than it had been while the patient was receiving antithyroid medication. PBI's were normal in all subjects, as were values for the daily degradation of organic iodine. Thyroidal uptakes of radioiodine greater than 50 per cent were found in three of six subjects, and were not necessarily associated with abnormally rapid peripheral turnover of thyroxine. Thyroidal uptake of radioiodine was suppressible in all instances.

Patients studied after surgical therapy

Values of the thyroxine half-time in the normal range were found in six of nine tests performed in the seven patients in this category. In the three tests in which abnormal values were

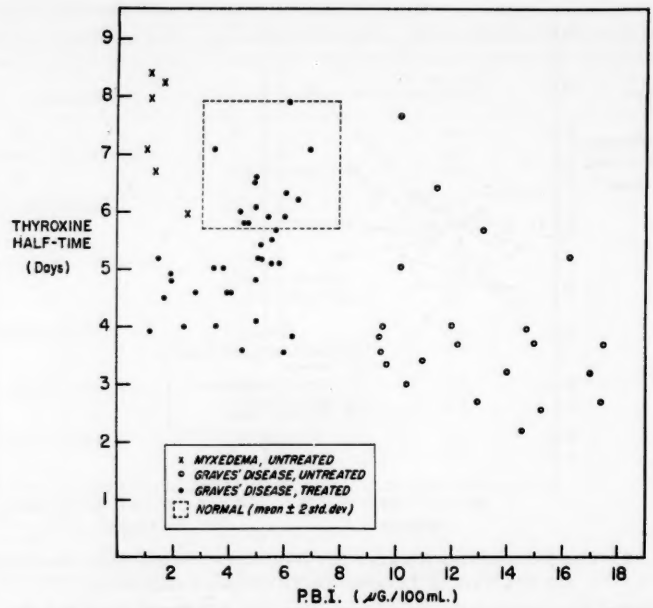

FIG. 2. THE RELATIONSHIP BETWEEN THE RATE OF TURNOVER OF THYROXINE AND THE CONCENTRATION OF CIRCULATING THYROID HORMONE IN PATIENTS WITH DIVERSE STATES OF THYROIDAL FUNCTION

Values in normal group based on observations in 30 normal subjects.

found, the duration of remission averaged 19 months. In the six instances in which normal values were found, the duration of remission averaged 6.3 years. PBI's averaged 4.9 ± 1.2 μg. per cent for the entire group and 4.1 μg. per cent in the three instances in which the turnover of thyroxine was rapid. Thyroidal uptake of radioiodine averaged 41 per cent at 24 hours in patients with rapid turnover of thyroxine and 33 per cent in patients whose thyroxine half-times were normal. Total daily degradation of organic iodine was normal in all subjects, and the thyroidal uptake of I^{131} could be suppressed by exogenous hormone in all instances.

Patients studied following miscellaneous therapy

One patient treated five years earlier with Lugol's solution as well as another patient treated six years earlier with radioactive iodine displayed accelerated peripheral turnover of thyroxine. PBI's, total daily degradation of hormone, and metabolic status were normal in both.

Effects of thyrotoxicosis medicamentosa (Table II)

Significant alterations in the kinetics of the metabolism of thyroxine were not seen in three patients given large doses of diiodotyrosine for periods of two months. On the other hand, significant acceleration of fractional turnover rate was induced in four patients made mildly thyrotoxic by the administration of either triiodothyronine or desiccated thyroid. Mean thyroxine half-time in this group decreased from 7.6 to 5.0 days. Administration of exogenous hormone was associated with a diminution in PBI in the three patients given triiodothyronine and a marked increase in PBI in the patient given desiccated thyroid. Daily degradation of organic iodine increased in the latter patient. In the patients who received triiodothyronine, calculations of the total quantity of organic iodine degraded daily could not validly be made, since neither the fraction of the PBI contributed by triiodothyronine nor its rate of turnover was known.

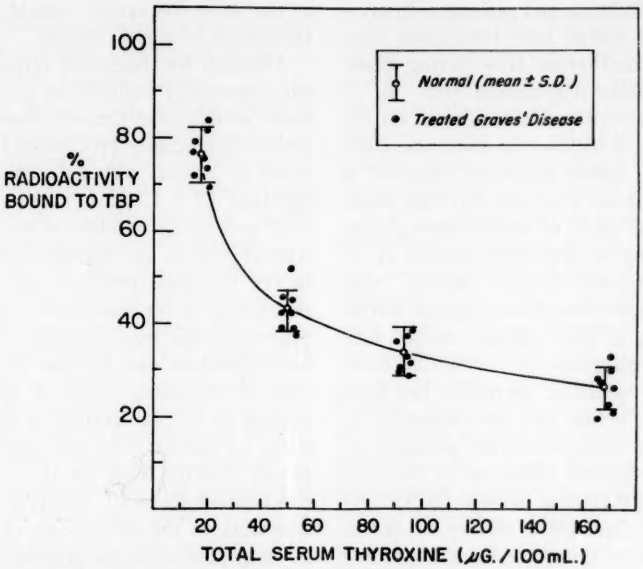

FIG. 3. BINDING OF THYROXINE BY TBP DURING ELECTROPHORESIS IN VERONAL BUFFER OF SERA OBTAINED FROM NORMAL SUBJECTS AND FROM PATIENTS WITH TREATED GRAVES' DISEASE

Concentrations of thyroxine in serum achieved by enrichment of serum with both stable and I^{131}-labeled hormone.

Thyroxine-binding by TBP

In eight patients with treated thyrotoxicosis who displayed persistent acceleration of thyroxine turnover, no abnormality of thyroxine-binding by TBP could be demonstrated when sera enriched with both stable and radioactive thyroxine were electrophoresed in veronal buffer, pH 8.6, ionic strength 0.05 (Figure 3).

Under similar electrophoretic conditions, thyroxine-binding by TBP was normal or increased in the sera of the three patients rendered hypermetabolic by the administration of triiodothyronine.

DISCUSSION

The majority of patients with untreated Graves' disease display an increase in the fractional rate of turnover of thyroxine in peripheral tissues (1–3). The present studies, intended to elucidate the factors responsible for this abnormality, have revealed that in a large number of patients, increase in fractional turnover rate may persist in greater or lesser degree for long periods following alleviation of the thyrotoxic state.[5] For the entire group of treated patients studied, thyroxine half-times averaged 5.2 ± 1.0 days, a value significantly different ($p < 0.001$) from the normal mean of 6.8 days (based on studies in 30 normal subjects). However, mean values of the PBI, daily degradation of organic iodine, and BMR were all within the normal range. Mean duration of remission averaged 28 months.

In order to obtain these measurements thyroidal reaccumulation of I^{131} was blocked by the administration of methyl-mercaptoimidazole. It seems reasonable to enquire whether this antithyroid agent might not be at least partially responsible for the augmentation of the rate of thyroxine turnover observed. This, however, seems unlikely. Since these studies were begun, all patients, in-

[5] Data concerning the turnover of thyroxine in patients with treated Graves' disease, somewhat at variance with the present findings, have recently been reported by Sterling (7). Since the data were presented in abstract form, attempts to reconcile differences in results must await fuller exposition of patient material and experimental method.

cluding normal individuals and patients with myxedema or nontoxic goiter, have been given comparable doses of antithyroid drug during determination of thyroxine degradation rate. In 21 normal subjects studied on this regimen, no difference in the rate of turnover of thyroxine could be discerned from values previously reported in 9 normal subjects studied without thyroidal blockade (1). Thus the effect of methyl-mercaptoimidazole, if any, must be selectively exerted in the population with treated Graves' disease. One pharmacological factor, however, requires further inquiry. In three of four patients studied with and without supplementation of antithyroid medication by Lugol's solution, thyroxine half-times were longer when iodine was concomitantly administered. These observations are perhaps correlated with the reported inhibition by iodide of the deiodination of thyroxine *in vitro* (8, 9). Although acute administration of iodides has not altered the half-time of thyroxine in nonthyrotoxic subjects (3), studies are now in progress to ascertain the effects, if any, of iodide on the turnover of thyroxine in patients with treated Graves' disease.

In the present studies, evaluation of the duration of accelerated turnover of thyroxine is rendered difficult by the variation in the mode of therapy employed. Thus, the majority of patients studied more than 3 years after treatment had undergone subtotal thyroidectomy, and displayed a normal rate of thyroxine turnover. On the other hand, the majority of patients studied during the first few years after onset of treatment had been treated with antithyroid drugs and displayed an abnormally rapid rate of thyroxine turnover. Despite the preponderance of abnormal values in patients treated with antithyroid drugs, the data do not support the supposition that the abnormally rapid turnover of thyroxine was somehow conditioned by these agents. Four of six patients continued to demonstrate the abnormality during sustained remission following cessation of antithyroid therapy, and abnormal values were found in the two patients treated with stable or radioactive iodine. Furthermore, among patients treated surgically, abnormally rapid turnover of thyroxine was observed in those studies performed less than three years after operation. These data suggest that time, rather than mode of therapy, is the more important variable with regard to restoration of normal values.

Although the fractional turnover of thyroxine was abnormally rapid in the present group of patients with treated Graves' disease, it was nevertheless significantly ($p < 0.001$) slower than that found in patients with active thyrotoxicity (mean half-time 3.9 ± 1.1 days, based on values obtained in 22 patients). In addition, in five of six patients studied prior to and during treatment, a reduction in the fractional turnover rate was noted after restoration of the eumetabolic state. Finally, the present studies have indicated that thyrotoxicosis medicamentosa can increase the fractional turnover of thyroxine. Thus, it seems likely that a portion of the acceleration of thyroxine turnover noted in patients with untreated Graves' disease results directly from the thyrotoxicosis or some concomitant thereof. However, the residual abnormality in the metabolism of thyroxine, noted in many patients in the present study, remained to be explained. No correlation was found between the presence or absence of increased fractional turnover of thyroxine and the age of the patient, or the presence of exophthalmos or of other associated endocrine or constitutional abnormalities. An explanation was further sought among those factors which have been suggested as possible determinants of the peripheral turnover of thyroxine. These have been extensively considered in an earlier communication (10), and will be considered here in relation to the present findings.

In serum, thyroxine is bound to specific transport proteins, one of which migrates during paper electrophoresis at pH 8.6 in the zone between the α_1 and α_2 globulins (11). This moiety has been termed the thyroxine-binding protein of plasma (TBP). Recently another highly potent thyroxine-binding protein has been noted in serum, having an anodal electrophoretic mobility greater than that of albumin (pre-albumin) (12). It has been suggested that the distribution and rate of turnover of thyroxine might be governed by the metabolism of the proteins to which it is bound, and it was initially suggested that the accelerated turnover of proteins and other metabolites which occurs in patients with Graves' disease might lead to acceleration of the turnover of the hormone (1). Although highly purified preparations of TBP and pre-albumin are now available (13), it has not as

yet been possible to ascertain whether the turnover of these proteins remains abnormally rapid in patients with treated Graves' disease. In any event, accelerated turnover of protein resulting from hypermetabolism, *per se,* would seem to be excluded as a contributory factor in the treated patient, who may continue to demonstrate rapid turnover of thyroxine despite reduction of the metabolic rate to normal or subnormal values.

Riggs (14), as well as Berson and Yalow (2), have suggested that the quantity of thyroxine degraded daily might vary linearly with the square of the PBI. No explanation for this relationship was apparent. More recently, however, Robbins and Rall have offered the following suggestion (15). These authors have hypothesized that in the plasma, thyroxine exists in equilibrium between the protein-bound and the free or unbound state. According to their calculations, values of free thyroxine vary inversely with the thyroxine-binding capacity of TBP and directly with the total concentration of hormone in the plasma. It was calculated that as the PBI increased, a progressively greater fraction of the plasma's hormone was present in the free state. Inspection of their data reveals that the calculated concentration of free thyroxine varies roughly with the square of the PBI. It is not surprising, therefore, that, employing the data of Berson and Yalow, Robbins and Rall found a linear relationship between the daily degradation of hormone and the concentration of free thyroxine.

Implicit in these considerations is the conclusion that the fractional rate of turnover of the total plasma hormone (the function measured in the present study) must vary with the concentration of free thyroxine as conditioned by *a*) the total concentration of hormone and *b*) the binding activity of TBP. In the present study, however, abnormally rapid fractional turnover of thyroxine has been found in patients whose PBI's were in the normal or the myxedematous range. Furthermore, since the sera of such patients demonstrated normal binding activity of TBP, calculated values of free thyroxine were normal or decreased. Thus, though the foregoing general relationships concerning thyroxine turnover may pertain in subjects with treated Graves' disease, they must at least be quantitatively altered, and the operation of other factors must be invoked.

Additional evidence in this regard is provided by the present patients made hypermetabolic with triiodothyronine. In these patients, PBI diminished and the binding capacity of TBP was unchanged. Here, too, calculated concentrations of free thyroxine were diminished, but the fractional rate of turnover of thyroxine was increased.

It is thus apparent that quantitative alterations in the interaction between thyroxine and TBP cannot account for the increased fractional rate of turnover of thyroxine found in some patients with treated Graves' disease. The role of interactions between thyroxine and pre-albumin has not as yet been evaluated. Preliminary studies indicate that the thyroxine-binding capacity of pre-albumin is markedly diminished during active thyrotoxicosis. Since binding of thyroxine by pre-albumin appears to be a critical determinant of the cellular uptake of thyroxine in *in vitro* systems, especially at concentrations of hormone in the thyrotoxic range (13), the reported increase in the cellular uptake of thyroxine *in vitro* from the sera of thyrotoxic patients (16, 17) may be merely a manifestation of altered binding by pre-albumin. Unfortunately, few data are available concerning cellular uptake of thyroxine from the serum of patients with treated Graves' disease, nor has it been possible as yet to assess the thyroxine-binding capacity of pre-albumin in a significant number of patients in this category.

Thus, the accelerated turnover of thyroxine found in some patients with treated Graves' disease cannot presently be correlated with any of the extracellular factors which have previously been suggested as possible determinants of the rate of turnover of the hormone. Attention may then turn to the possible role of factors within the cell itself. As has been suggested elsewhere (10), alterations in the activity of those cellular components which bind thyroxine might be expected to augment the rate of removal of hormone from the extracellular fluid, and, if subsequent steps in the metabolism of hormone were not rate-limiting, might result in increased fractional rate of turnover of hormone. At present, however, techniques are not available for assessing this variable.

Enzymes capable of degrading thyroxine have been demonstrated in a variety of tissues by a number of investigators (8, 9, 18). Larson, Tomita and Albright have noted an increase in

the thyroxine-deiodinating activity of renal tissue of rats made hypermetabolic by the administration of thyroxine, and have suggested that this may represent an adaptive augmentation of enzyme activity, consequent upon an increased delivery of substrate (19). A similar sequence could occur in patients with active Graves' disease, and could be responsible, at least in part, for the accelerated turnover of thyroxine found in untreated patients. However, currently available data provide no basis for prediction of how long such adaptive increases in the deiodination of thyroxine might persist following alleviation of the hyperthyroxinosis. Persistence for as long as five or six years, the duration of the most persistent abnormalities in turnover noted in the present study, seems highly unlikely. The present data further indicate that if adaptive increases in deiodination do occur, they must be relatively substrate specific, since administration of large quantities of diiodotyrosine for periods of two months failed to alter the rate of turnover of thyroxine. The acceleration of thyroxine turnover found in patients made hypermetabolic with triiodothyronine might be construed as indicating that the two hormones share common deiodinating pathways. Alternatively, it seems possible that in these patients, as in rats given large doses of thyroxine, acceleration of hormonal degradation resulted from hypermetabolism, *per se*.

Thus, it is difficult to ascribe the increased fractional turnover of thyroxine found in some patients with treated Graves' disease to any of the several mechanisms which have already been suggested as possible regulators of the peripheral turnover of the hormone. However, other possibilities remain to be considered. Among these, consideration must be given to the thyroid gland itself. In the present series, many, though not all of the patients who demonstrated abnormally large fractional rates of turnover of thyroxine also displayed 24 hour thyroidal uptakes of I^{131} well above the normal range. This was most commonly found in patients receiving antithyroid drugs. Administration of small quantities of iodine failed to diminish the increased uptakes, indicating that iodine deficiency was not responsible for the augmentation of uptake. In the several instances in which KSCN was administered, only a small fraction of the accumulated iodine could be discharged from the gland. Thus, it may be concluded that despite the antithyroid drugs, large quantities of iodine were being incorporated into organic moieties. Moreover, by direct counting over the neck, the rate of release of iodine from the thyroid gland of these patients was found to be rapid. Therefore, these organic moieties were being lost from the gland at a rapid rate. Measurements of protein-bound radioiodine in the plasma were not made. It is thus not possible to be certain whether the rapid disappearance of radioactivity from the gland represented intrathyroidal deiodination or secretion into the circulation. However, the normal values for PBI and BMR which were observed in these instances would suggest that if such material were indeed reaching the circulation, it was rapidly removed and had little calorigenic potency. Iodinated tyrosyl derivates, such as diiodotyrosine (DIT) and monoiodotyrosine (MIT) could fulfill these criteria. Recent studies indicate that when organic-binding of iodine in the thyroid glands of rats is blocked by increasing doses of propylthiouracil, there occurs a progressive diminution and ultimately a complete disappearance of I^{131}-labeled thyronines, associated with a progressive reduction in the DIT/MIT ratio, until MIT becomes the sole radioiodinated compound demonstrable (20). Thus, it seems possible that MIT, DIT, or both, may be released from highly active, partially blocked glands, in which the rate of proteolysis and release may exceed the capacity of glandular dehalogenase. In an earlier study, evidence was presented which suggested that compounds of this type may be released from the thyroid gland of the patient with Graves' disease, even prior to the institution of therapy (1), and subsequently iodotyrosines were demonstrated chromatographically in the serum of patients with Graves' disease (21). It is conceivable that such iodinated compounds might stimulate peripheral deiodinating mechanisms. The present negative results with DIT would not support this hypothesis. Nonetheless, it remains possible that other, as yet untested compounds, capable of augmenting the peripheral turnover of thyroxine, may be released from the gland of the treated patient.

Consideration must also be given to the possibility that the thyroid gland itself contributes to the degradation of thyroxine in the patient with

treated Graves' disease. In small animals, exogenous thyroxine does not penetrate the thyroid gland, unless antithyroid agents, such as propylthiouracil are concomitantly administered (22). The patients in the present series were all given blocking doses of Tapazole® during study periods. It is therefore possible that a portion of the administered I^{131}-labeled thyroxine did penetrate the thyroid gland in these patients, although appreciable thyroidal radioactivity could not be demonstrated by direct counting. Whether thyroxine, once having penetrated the gland, would be metabolized is entirely unknown. Although mechanisms for the intrathyroidal deiodination of thyroxine have not been demonstrated in the normal gland, their possible presence in the gland of the patient with Graves' disease has not been excluded.

Thus, the thyroid gland could contribute to the peripheral degradation of thyroxine, either by secreting a product which stimulates deiodination or by itself degrading the hormone. In an effort to evaluate these possibilities, the effects of prolonged thyroidal suppression on the peripheral degradation of thyroxine are now under investigation.

The possibility remains that the abnormally rapid peripheral turnover of thyroxine in some way reflects the activity of the disease process *per se*. Persistence of the abnormality in the treated state might then reflect the continued operation of underlying pathogenetic mechanisms. It is of interest in this regard that of the 11 patients whose last recorded value of the thyroxine half-time was in the abnormal range, 2 patients have died of unrelated causes, and 3 (Nos. 1, 5, 7) have experienced recurrence of active thyrotoxicity. Of the 12 patients whose last recorded values were within the normal range, none are known to have relapsed clinically. Additional evidence in this regard is perhaps afforded by the observation that accelerated turnover of thyroxine is found in a significant proportion of relatives of patients with Graves' disease, who are not themselves and apparently have never been clinically thyrotoxic (23).

Another abnormality of iodine metabolism has been thought to reflect activity of the morbid process in Graves' disease. This is the failure of exogenous thyroid hormone, even in large doses, to suppress the thyroidal uptake of radioactive iodine (24, 25). This abnormality, like the increased fractional turnover of thyroxine peripherally which has been described above, has been reported by Werner to persist for long periods after alleviation of active thyrotoxicity (26). Moreover, like the abnormality of peripheral turnover, it frequently abates several years after treatment. In the present studies, no correlation was found between the presence or absence of thyroidal suppression by exogenous hormone and the presence of accelerated fractional turnover of thyroxine. The present data indicate that thyroidal suppression can commonly be achieved while patients are receiving antithyroid therapy. In patients treated by other means, and in patients observed following cessation of antithyroid therapy, the incidence of resistance to suppression in the present series seems less than that reported by Werner (26), and more consistent with the data of Morgans, Oldham and Trotter (27). It seems quite clear, at any rate, that the currently described abnormality is not of sufficient magnitude to account for resistance to suppression by large doses of exogenous hormone, based on increased disposal of the administered material. The precise relationship of the two abnormalities to each other and to the underlying pathogenesis of Graves' disease remains to be resolved in the future.

SUMMARY

1. Studies of the distribution and rate of peripheral degradation of exogenous I^{131}-labeled thyroxine have been performed in 23 patients with Graves' disease, following restoration of a eumetabolic status.

2. Among these patients, many displayed a persistence of increased fractional rate of turnover of the hormone in peripheral tissues for long periods after induction of symptomatic remission.

3. Although induction of thyrotoxicosis medicamentosa by the administration of either desiccated thyroid or triiodothyronine has been shown to increase the fractional rate of turnover of thyroxine, hypermetabolism, *per se*, cannot account for the increase noted in the patients with treated Graves' disease.

4. Analysis of several other factors which might influence the peripheral turnover of thyroxine has failed to reveal the cause of the abnormality in these patients.

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AMMONIA EXCRETION AND RENAL ENZYMATIC ADAPTATION IN HUMAN SUBJECTS, AS DISCLOSED BY ADMINISTRATION OF PRECURSOR AMINO ACIDS [1, 2]

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Urinary ammonia is produced in renal tubular cells by the deamidation of glutamine and asparagine and the deamination of a variety of amino acids (1–5). Many ammonia-producing enzymes have been identified in mammalian kidney and both their presence and activity appear to vary widely with species (5–14). In the dog, 60 per cent of the urinary ammonia has been attributed to the deamidation of serum glutamine, the remaining 40 per cent apparently originating from amino acid oxidase activity (1). In the human kidney, glutaminase activity is only 0.1 to 0.01 that present in dogs (15). Renal *l*-amino acid oxidase activity is low or absent in most species save the rat (8) and renal *d*-amino acid oxidase varies widely in different species, the activity in the cat being 20 times that in the guinea pig (14).

Recently it has been shown in rats that strong acid loads increase renal ammonia production consequent to adaptation of glutaminase (16–19), glycine oxidase and *l*-amino acid oxidase (16). Increased renal ammonia production and excretion also follows the administration of precursor amino acids; in dogs the administration of a *dl*-alanine, *l*-leucine, glycine, glutamine and *dl*-aspartic acid results in a prompt increase in urinary ammonia excretion (1–4).

It is not known precisely which ammonia-producing enzymes are present in the human kidney and which of these undergo adaptive change during the administration of strong acid loads. The purposes of this study were threefold: to determine which amino acids are precursors of urinary ammonia, thereby furnishing evidence for the presence of various ammonia-producing enzyme systems in the human kidney, to ascertain which of these enzymes show adaptive increase in activity and finally to help define the relationship between ammonia production and ammonia transport by observing the changes in urinary acid excretion during the administration of precursor amino acids.

PROCEDURE

Five normal male subjects, housed on the metabolic ward and maintained on a diet of constant composition, were given NH_4Cl loads continuously for from one to five months. One hundred twenty-five studies, each lasting 8 to 10 hours, were performed. To identify the amino acid precursors of urinary ammonia, the subjects were kept on 15 Gm. of ammonium chloride until ammonia excretion was constant. Single amino acids (230 to 400 mM) were then given orally over a 60 to 90 minute period and changes in urinary ammonia, pH and titratable acid (TA) were followed hourly for six to eight hours. These values then were compared to those obtained during a control period of equal duration, performed during the same hours of the day in order to obviate changes related to diurnal variation.

To determine which renal enzyme systems adapt to strong acid loads, subjects were studied under three circumstances: standard diet (SD) plus 5 Gm. of NH_4Cl per day; SD plus 10 Gm. NH_4Cl per day; and finally, SD plus 15 Gm. NH_4Cl per day. Each level of NH_4Cl administration was continued from 10 to at least 30 days. When a steady state was attained, as evidenced by a constant urinary ammonia excretion during a fixed six to eight hour period each day (9:00 a.m. to 5:00 p.m.), equimolar amounts (300 mM) of different amino acids were given on different days.[3] Experiments were performed at no less than three day intervals to allow adequate time for return to a steady state. The amino acids, dissolved in 500 to 1,000 ml. of water, were administered orally over a 60 to 90 minute period. Urine specimens were collected hourly under mineral oil in bottles containing phenyl mercuric nitrate as a preservative. Im-

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[2] Presented at the meeting of the American Federation for Clinical Research in Atlantic City, May, 1955.

[3] On the days of the amino acid loading and control experiments, NH_4Cl was not administered until after the study was completed. The subjects therefore did not receive NH_4Cl for 14 hours prior to each experiment.

TABLE I

Mammalian renal enzyme systems capable of ammonia production *

ENZYME	SUBSTRATE	PRODUCTS
1. Glutaminase [5,12,15]	Glutamine	Glutamic acid + ammonia
2. Asparaginase [12]	Asparagine	Aspartic acid + ammonia
3. D-Amino acid oxidase [14]	Most D-amino acids	Corresponding α-keto acid + ammonia
4. L-amino acid oxidase [7,8,14]	Most L-amino acids	Corresponding α-keto acid + ammonia
5. Glycine oxidase [6]	Glycine	Glyoxylic acid + ammonia
6. Glutamic acid dehydrogenase [14]	L-glutamic acid	α-ketoglutaric acid + ammonia
7. Transaminase [13]	a. L-aspartic acid + α-ketoglutaric acid	Oxaloacetic acid + glutamic acid
	b. L-alanine + α-ketoglutaric acid	Pyruvic acid + glutamic acid
8. Proline oxidase [10,14]	Proline	L-glutamic acid

* Neither the transaminases nor proline oxidae directly produce ammonia but if coupled with gluatamic dehydrogenase, a net production of ammonia occurs. The superscript indicates a reference number.

mediately after voiding, ammonia, TA and pH were determined. In 42 experiments titratable acid minus bicarbonate ($TA-HCO_3$) was measured. The values obtained on experimental days, when amino acid was given, at each level of NH_4Cl administration, were compared to the values obtained on control days at the same level of NH_4Cl administration. The *net change* (experiment minus control) in ammonia excretion, attributable to the administration of each amino acid at each level of NH_4Cl administration, was thus obtained. The *net change* in urinary ammonia [or ammonia plus ($TA-HCO_3$)] after a specific amino acid, while the subject was receiving 5

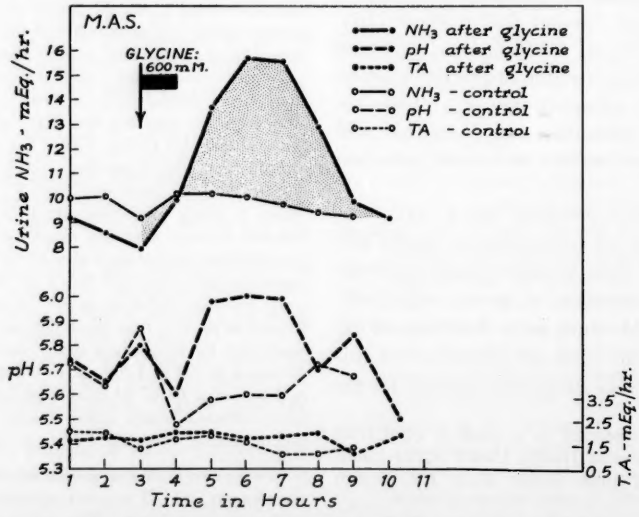

FIG. 1. EFFECT OF ADMINISTRATION OF GLYCINE ON URINARY AMMONIA EXCRETION, pH AND TITRATABLE ACID

Gm. NH_4Cl per day, was then compared to the net change that occurred when the subject received 10 and then 15 Gm. NH_4Cl per day.

Methods. Urinary ammonia was determined in duplicate by a modification of the microdiffusion method of Conway (20). Urine pH was measured with a Beckman pH meter using external glass electrodes and corrected to 37.5° in accord with the findings of Wesson for human urine (21). Urine was titrated to pH 7.4 with 0.1 N NaOH to determine titratable acid. TA–HCO_3 was measured by the method of Dawson, Dempsey, Bartter, Leaf and Albright (22). Serum CO_2 content was determined manometrically (23). Serum chloride was measured by the method described by Peters and Van Slyke (23); serum sodium and potassium concentrations were determined on the flame photometer (24).

RESULTS

1. *Identification of ammonia-producing enzyme systems in human kidney by administration of precursor amino acids*

The enzymes producing ammonia from amino acids which have been identified *in vitro* in mammalian kidney are listed in Table I. A typical experiment showing an augmented ammonia excretion after administration of glycine to a subject stabilized on 15 Gm. NH_4Cl daily is presented diagramatically in Figure 1. The prompt rise in urinary ammonia excretion lasting four to six hours suggests that glycine oxidase is present in human kidney. The results after the administration of 12 different amino acids suggest that all

TABLE II

*Protocols of typical amino acid loading and control studies.**

	CONTROL							EXPERIMENT						
PREPARATION	AMINO ACID	SERUM CO_2 CONTENT mEq/L.	TIME minute	URINE VOL. cc	pH	NH_3 mEq/TV	T.A. mEq/TV	AMINO ACID	SERUM CO_2 CONTENT mEq/L.	TIME minutes	URINE VOL. cc.	pH	NH_3 mEq/TV	T.A. mEq/TV
Standard diet + 5 gm. NH_4Cl day	none	26.2	0-60	269	5.32	4.2	1.8	Glycine 400mM	27.9	0-60	129	5.12	3.8	2.9
			60-120	140	5.18	3.4	1.9			60-120	118	5.77	5.2	2.4
			120-180	173	5.19	4.0	2.3			120-180	203	6.02	6.2	2.0
			180-240	239	5.38	3.1	2.0			180-240	270	5.72	4.8	2.0
			240-300	135	5.20	3.2	1.8			240-300	297	5.53	3.5	2.4
			300-360	195	5.23	4.3	2.2			300-360	169	5.18	3.1	2.8
					TOTAL	22.2	12.0					TOTAL	26.6	14.5
Standard diet + 10 gm. NH_4Cl q.d.	none	23.4	0-60	314	5.19	7.3	2.7	Glycine 400mM	24.0	0-60	105	5.18	5.9	1.3
			60-120	222	5.19	5.9	2.6			60-120	190	5.67	16.1	2.8
			120-180	224	5.13	6.1	2.4			120-180	160	5.95	11.6	1.4
			180-240	140	5.02	5.4	1.4			180-240	174	6.17	9.4	1.1
			240-300	170	5.12	5.5	1.7			240-300	140	5.97	7.4	1.1
			300-360	130	5.19	4.5	1.6			300-360	140	5.58	5.8	1.8
					TOTAL	34.6	12.4					TOTAL	56.2	9.5
Standard diet + 15 gm. NH_4Cl q.d.	none	20.6	0-60	140	5.46	11.6	2.4	Glycine 400mM	20.7	0-60	55	5.78	9.1	1.4
			60-120	75	5.60	7.8	2.0			60-120	152	6.12	17.1	2.2
			120-180	120	5.55	9.2	1.2			120-180	68	6.08	7.3	0.7
			180-240	110	5.45	8.9	2.1			180-240	226	6.15	30.5	1.7
			240-300	112	5.49	9.7	1.3			240-300	499	6.42	13.7	1.8
			300-360	87	5.60	8.7	1.4			300-360	226	6.18	19.4	1.4
					TOTAL	55.9	10.4					TOTAL	97.1	9.2

* TV refers to total urine volume.

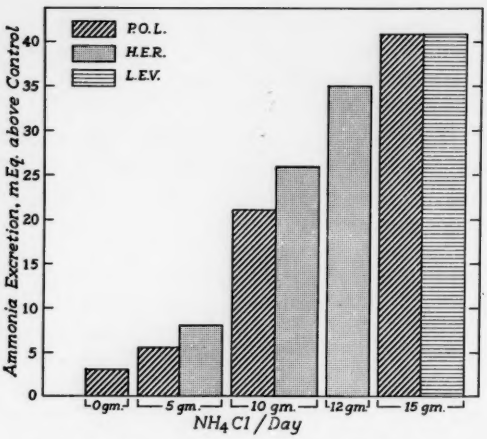

FIG. 2. EFFECT OF GLYCINE ADMINISTRATION ON NET AMMONIA EXCRETION IN THREE SUBJECTS DURING STEPWISE INCREASE IN THE MAGNITUDE OF THE CHRONIC AMMONIUM CHLORIDE LOADS

of the enzyme systems listed in Table I are present in human kidney. The administration of *l*-lysine did not increase ammonia excretion in keeping with similar findings in dogs (*2*).

2. Adaptation of ammonia-producing enzymes in the human kidney during the chronic administration of ammonium chloride loads

The protocols from a typical series of experiments performed to determine whether enzyme adaptation occurred are shown in Table II. *Net ammonia excretion* (experiment minus control) progressively increased from 4.2 to 21.6 to 41 mEq. following the administration of 400 mM of glycine to a subject chronically maintained on 5, then 10 and finally 15 Gm. of NH_4Cl per day. Data from three subjects showing the increasing magnitude of net ammonia excretion following the administration of 400 mM of glycine at different levels of NH_4Cl intake are shown in Figure 2.

Typical examples of the results of experiments in two other subjects performed in a similar manner to determine whether or not adaptation of glutaminase, asparaginase, *d*-amino acid oxidase and *l*-amino acid oxidase occurred are shown in Figures 3 and 4.

The mean increase in net ammonia excretion for the five subjects following the administration of this group of precursor amino acids (glycine,

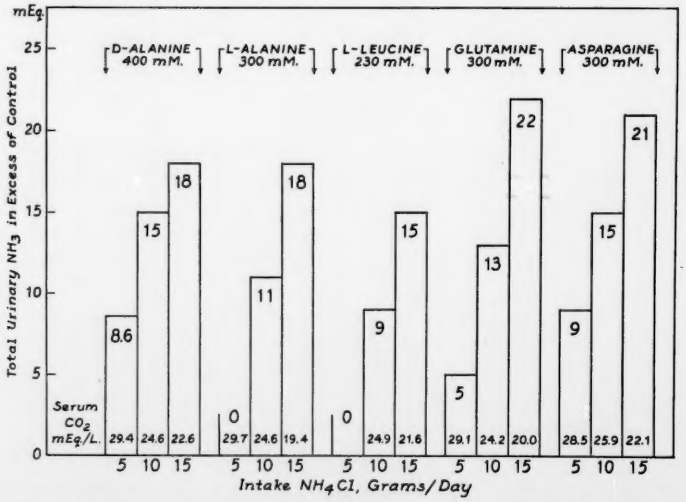

FIG. 3. EFFECT OF DIFFERENT AMINO ACIDS AND AMINO ACID AMIDES ON NET URINARY AMMONIA EXCRETION FOLLOWING STEPWISE INCREASE IN THE MAGNITUDE OF THE CHRONIC AMMONIUM CHLORIDE LOAD

The numbers in the columns refer to the net increase in urinary ammonia after the substrate load.

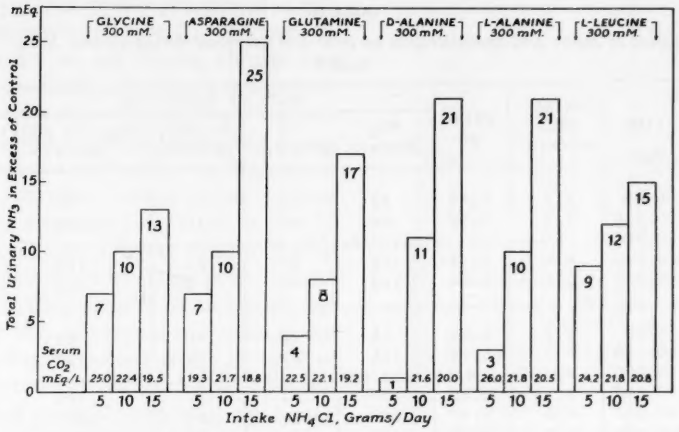

FIG. 4. EFFECT OF DIFFERENT AMINO ACIDS AND AMINO ACID AMIDES ON NET URINARY AMMONIA EXCRETION FOLLOWING STEPWISE INCREASE IN THE MAGNITUDE OF THE CHRONIC AMMONIUM CHLORIDE LOAD

The numbers in the columns refer to the net increase in urinary ammonia after the substrate load.

asparagine, glutamine, *l*-alanine, *d*-alanine and *l*-leucine) increased significantly ($p = < 0.01$) from 4.8 to 12.2 to 20.6 mEq. as the chronic maintenance dose of NH_4Cl was increased from 5 to 10 to 15 Gm. a day.

By contrast, the administration of glutamate, aspartate or proline resulted in only a small relatively fixed net increment in ammonia excretion (2 to 7 mEq.) despite increasing magnitude of the ammonium chloride loads (Figure 5). To be

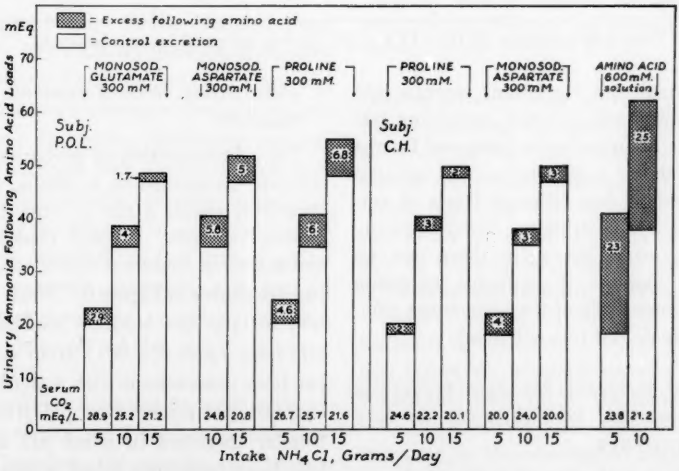

FIG. 5. EFFECT OF GLUTAMATE, ASPARTATE AND PROLINE ON NET URINARY AMMONIA EXCRETION FOLLOWING STEPWISE INCREASE IN THE MAGNITUDE OF THE CHRONIC AMMONIUM CHLORIDE LOAD

The numbers in the columns refer to the net increase in urinary ammonia after the substrate load.

TABLE III

Effect of amino acid administration on total acid excretion during chronic NH_4Cl loads *

TIME min.	URINE VOL. ml./min.	URINE pH	RATE OF EXCRETION			
			NH_3 μEq/min.	TA μEq/min.	$TA-HCO_3$ μEq/min.	$NH_3+(TA-HCO_3)$ μEq/min.
0-60	3.7	5.68	87	14	12	99
60-120	2.7	5.52	84	15	14	98
120-200	l-alanine 300 mM					
120-240	6.5	5.77	159	27	23	182
240-360	3.4	6.04	193	12	9	202
0-60	3.2	5.56	84	13	11	95
60-120	4.9	5.34	114	21	19	133
120-200	1500cc. amino acid solution (600mM)-12.5cc/min.					
120-240	5.1	5.65	164	23	22	185
240-360	4.0	5.90	200	24	21	221
360-480	3.5	5.73	156	17	15	171
0-60	1.2	5.80	115	16	15	130
60-120	1.1	5.85	126	17	15	141
120-200	l-asparagine 300 mM					
120-240	2.7	6.00	215	26	24	239
240-360	7.0	6.39	253	21	18	273
360-480	1.7	6.00	136	18	17	153
0-60	1.2	5.74	135	29	20	155
60-120	0.8	5.80	96	20	18	114
120-200	D-alanine 300 mM					
120-240	3.8	5.89	177	36	23	200
240-360	6.0	6.10	234	16	15	249
360-480	8.0	5.80	155	44	43	198

* Total acid excretion $[NH_3 + (TA - HCO_3)]$ is increased in each instance.

certain that a small but significant increase did not occur, an amino acid solution containing 600 mM of a mixture of amino acids composed for the most part of glutamic acid, proline and aspartic acid [4] was infused at two different levels of ammonium chloride administration. Total ammonia excretion was greater, but again there was no progressive net increase in ammonia excretion with increasing magnitude of the ammonium chloride load (Figure 5, last two columns).

3. The effect of increased ammonia production following amino acid administration on total urinary acid excretion

The invariable and significant augmentation of total acid excretion [NH_3 plus ($TA-HCO_3$)] after amino acid loads is indicated by the four typical protocols listed in Table III.

[4] Approximately 25 mM each of serine, valine, phenylalanine and threonine were also present.

4. Relationship between ammonia production and transport

The administration of precursor amino acid to subjects maintained on a chronic strong acid load invariably causes a rise in urine pH as NH_3 excretion increases. Typical changes in urine pH and ammonia excretion which occured in one subject are shown in Figure 6. Mean ammonia excretion for the five subjects studied increased significantly ($p = < 0.001$) from 6.1 to 10.1 mEq. per hour concomitant with a significant rise ($p = < 0.001$) in mean urine pH from 5.60 to 6.12. Similar increases in urine pH following amino acid loads have been found in dogs by some (25) but not by others (2).

When subjects continuously received NH_4Cl it was noted that, after a steady state was attained at each level of NH_4Cl administration, urinary ammonia increased as the magnitude of the chronic

acid load increased but urinary pH, instead of falling, rose progressively (Figure 7). In order to determine whether or not, during chronic acid loads, urinary ammonia excretion is independent of changes in urine pH, acute alkalosis was produced by the infusion of sodium bicarbonate during a time when ammonia production was being stimulated toward maximal levels. One subject (M. A. S.) was maintained on 15 Gm. of NH_4Cl a day for about 30 days. At this time 600 mM of glycine was administered, resulting in an increase in ammonia excretion from 8 to 15.6 mEq. per hour. The identical experiment was repeated but in addition during the second study 250 mM of $NaHCO_3$ was infused producing an acute alkalosis, serum CO_2 rising from 22 to 35 mEq. per L. and urine pH from 5.59 to 7.63. The expected increase in ammonia excretion incident to the anticipated increase in ammonia production was not forthcoming. Instead urinary ammonia excretion fell progressively from 8.2 to 2.4 mEq. per hour. These experiments are compared in Figure 8.

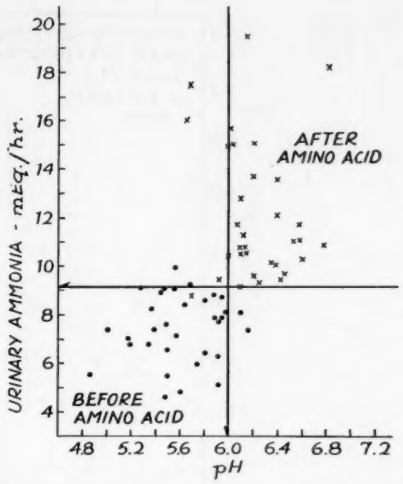

FIG. 6. RELATIONSHIP BETWEEN URINE pH AND AMMONIA EXCRETION BEFORE AND AFTER THE ADMINISTRATION OF AMINO ACID LOADS

The solid dot represents the peak ammonia excretion with associated urine pH prior to amino acid administration and the cross, the peak urinary ammonia and associated urine pH after amino acid administration. In each instance ammonia excretion and urine pH increased after amino acid administration. The horizontal and vertical lines are arbitrarily drawn to maximally separate the values before and after amino acid loading. In this subject mean ammonia excretion increased from 7.5 to 12.2 mEq. per hour despite a rise in mean urine pH from 5.6 to 6.2.

DISCUSSION

The administration of an amino acid during the chronic maintenance of NH_4Cl loads of increasing magnitude results in three types of response: *1*) *l*-Lysine and presumably similar diaminomonocarboxylic acids (*2*) elicit no increase in urinary ammonia excretion; *2*) glutamate, aspartate and proline result in a small, constant increment in ammonia excretion; *3*) glycine, glutamine, asparagine, *l*-alanine, *l*-leucine and *d*-alanine each progressively augment ammonia excretion in stepwise fashion paralleling the increase in ammonium chloride load. These data indicate that certain amino acids augment ammonia excretion, and also in the light of certain theoretical considerations help to identify the character of the renal enzymatic adaptation.

These experiments, designed to determine *in vivo* adaptation of the renal ammonia-producing enzymes, were based on the premise that at any given substrate concentration more end-product will be formed per unit time if more enzyme is present. If the chronic administration of strong acid loads results in an increase in renal tubular enzyme activity,[5] then the renal production of ammonia following the same quantity of amino acid substrate before and after enzyme adaptation should differ quantitatively. After enzyme adaptation, the identical substrate load should result in an increased ammonia production ascribable to the increased enzyme activity. This hypothesis is schematically represented in Figure 9.

Before enzyme adaptation, as substrate concentration is increased from S^1, the plasma concentration before the administration of the fixed single amino acid load, to S^2, the plasma concentration after the substrate load, ammonia production per unit time by the nonadapted enzyme E^1 increases from P^1 to P^2. The *net increase* in ammonia pro-

[5] The increased enzyme activity that attends the adaptive process may be the result of either increased enzyme concentration, increased enzyme activator or decreased enzyme inhibitor.

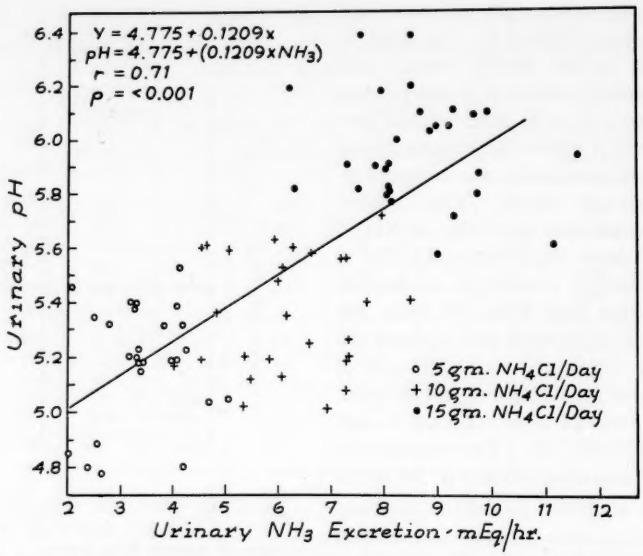
Y = 4.775 + 0.1209x
pH = 4.775 + (0.1209 x NH3)
r = 0.71
p = <0.001
Urinary pH
Urinary NH3 Excretion - mEq/hr.
o 5 gm. NH4Cl/Day
+ 10 gm. NH4Cl/Day
• 15 gm. NH4Cl/Day

FIG. 7. RELATIONSHIP BETWEEN AMMONIA EXCRETION AND URINARY pH DURING CHRONIC NH_4Cl LOADS

As the chronic acid load was increased in stepwise fashion mean urinary ammonia excretion increased from 3.4 to 6.2 to 8.6 mEq. per hour. Urinary pH also increased progressively from 5.18 to 5.39 and reached 5.96 as the NH_4Cl load was increased from 5 to 10 to 15 Gm. per day.

duction ascribable to the effect of the substrate load on enzyme E^1 is therefore equal to (P^2 minus P^1). If the activity of the ammonia producing enzyme increases from E^1 to E^2 after the administration of a strong acid load, then the administration of the same quantity of substrate should result in a greater *net increase* in ammonia production (P^4 minus P^3) compared to the unadapted enzyme (E^1). Finally, since the magnitude of the *net increase* in ammonia production after a substrate load is dependent on the enzymatic activity, the ratio (P^4 minus P^3)/(P^2 minus P^1), should reflect the magnitude of change in enzyme activity from E^1 to E^2.

In applying this hypothesis to *in vivo* experiments two assumptions were made:

1) The quantity of amino acid substrate delivered to the tubular enzymes per unit time after an identical substrate load does *not* progressively increase as the magnitude of the ammonium chloride load is increased from 5 to 15 Gm. per day. The renal hemodynamic changes that accompany the administration of ammonium chloride loads indicate a decrease in glomerular filtration rate and renal plasma flow (*27*). A decrease in the delivery of substrate with increasing NH_4Cl loads would not interfere with the interpretataions since it would tend to minimize, not maximize, the magnitude of the adaptive process. Since the amino acids were administered orally, traversing the liver prior to entry into the systemic circulation, the plasma concentration is, in part, dependent upon the fate of the amino acid in the liver. It is possible that as the magnitude of the NH_4Cl load was progressively increased, the larger doses may have increasingly loaded the hepatic urea-synthesizing mechanism, thereby permitting larger amounts of amino acid to escape to the systemic circulation. Were this true, then the increasing ammonia excretion following identical oral substrate loads administered at high levels of NH_4Cl intake, could represent increasing substrate reaching the kidney rather than enzyme adaptation; this seems unlikely for two reasons. One, the subjects did not

receive any NH_4Cl for 14 hours prior to control or amino acid loading studies (see Procedure). Moreover when 600 mM of asparagine was administered to a subject maintained on 5 Gm. of NH_4Cl per day, net ammonia excretion was 15 mEq. By contrast when maintained on 15 Gm. of NH_4Cl, the same subject's net ammonia excretion after only 300 mM of asparagine was 25 mEq. In another subject maintained on 5 Gm. of NH_4Cl, net ammonia excretion after 600 mM of glycine was 14 mEq. When maintained on 10 and 15 Gm. of NH_4Cl an intake of only 400 mM of glycine resulted in a net ammonia excretion of 22 and 41 mEq., respectively.

2) It must also be assumed in these experiments that ammonia *excretion* quantitatively reflects the magnitude of renal ammonia *production*. Ammonia produced within the tubular cells has three different fates, *i.e.*, excretion into tubular urine; diffusion into renal venous blood; and intracellular utilization. Despite these alternative pathways, the present analysis is not adversely affected since the bulk of the ammonia formed in acidotic animals is excreted (1, 25) and, therefore, although not equal to, reflects quantitatively ammonia production. In support of this assumption are other studies from this laboratory in rats given increas-

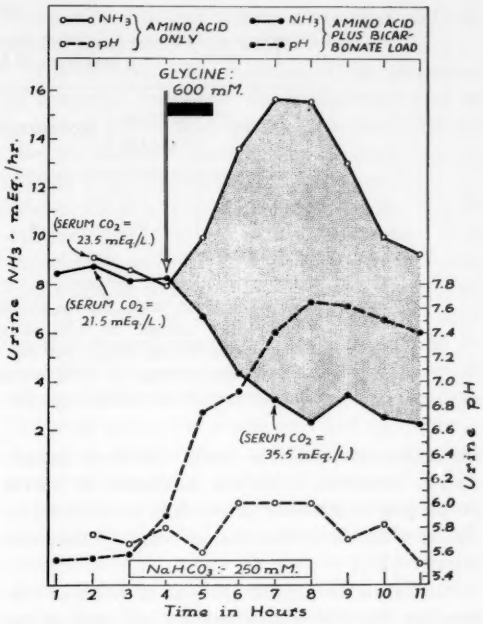

FIG. 8. EFFECT OF ACUTE ALKALOSIS ON URINARY AMMONIUM EXCRETION AFTER AMINO ACID ADMINISTRATION

See text for details.

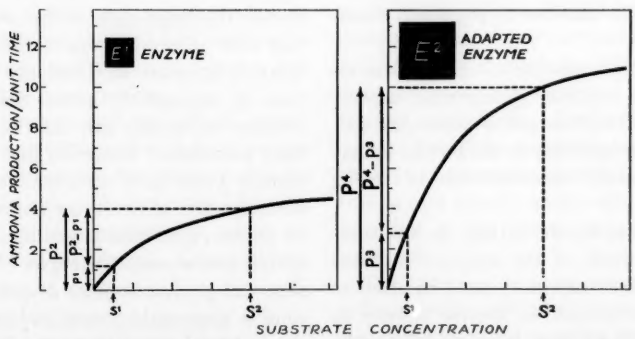

FIG. 9. EFFECT OF ENZYME ADAPTATION ON THE MAGNITUDE OF AMMONIA PRODUCTION FOLLOWING A FIXED SUBSTRATE LOAD

The conditions before enzyme adaptation (E^1) are shown on the left and after enzyme adaptation (E^2) on the right. Substrate concentration in arbitrary units is plotted along the abscissa and the rate of reaction, in arbitrary units of end-product formed per unit time, along the ordinate. The curved line in each panel describes the effect of increasing substrate concentration on the rate of reaction. The steeper curve on the right is the result of the increased activity of the adapted enzyme (26). See text for details.

TABLE IV

Comparison of the mean increase in enzyme activity after increasing the NH_4Cl load from 10 to 15 Gm. per day*

ENZYME	GLYCINE OXIDASE	GLUTAMINASE	ASPARAGINASE	l-AMINO ACID OXIDASE	d-AMINO ACID OXIDASE
$\frac{(P^6-P^5)^{\dagger}}{(P^4-P^3)^{\ddagger}}$	1.6	1.9	1.9	1.9	1.5

* Refers to mean values for the five subjects.

† Equals net increase in NH_3 excretion after substrate load during chronic maintenance on 15 Gm. NH_4Cl per day.

‡ Equals net increase in NH_3 excretion after substrate loads during chronic maintenance on 10 Gm. NH_4Cl per day.

ing ammonium chloride loads. In those experiments, ammonia excretion increased in direct proportion to ammonia production, as reflected by the *in vitro* determination of renal glutaminase activity (19).

Finally, in the present study at all levels of ammonium chloride intake, urinary pH was in the acid range. Moreover, mean urinary pH increased as the magnitude of the chronic ammonium chloride load was increased (Figure 7). With such changes in urinary pH, a progressively increasing net ammonia excretion in response to a fixed substrate load cannot be ascribed to improved conditions for transport.

In the light of these considerations, the data indicate that glycine oxidase, glutaminase, asparaginase, *l*-amino acid oxidase and *d*-amino acid oxidase show adaptive increases in the human kidney following the chronic administration of strong acids.

Although the data do not permit the measurement of the magnitude of the adaptive response of each of these five enzyme systems in absolute terms, the *relative* increase in enzyme activity as the size of the NH_4Cl load is increased can be estimated. Since the magnitude of the *net increase* in ammonia production after a substrate load is dependent on the enzyme activity, then the ratio, *net increase in ammonia production after substrate during maintenance on 15 Gm. NH_4Cl per day/ net increase in ammonia production after identical substrate load during maintenance on 10 Gm. of NH_4Cl per day,* should reflect the magnitude of increase in enzyme activity as the chronic load of NH_4Cl was increased from 10 to 15 Gm. per day.[6] These changes for each of the enzymes which undergo adaptive increase are compared in Table IV. The ratio of change for this group of enzymes varied from 1.5 to 1.9, indicating a one and a half to twofold increase in enzyme activity as the chronic NH_4Cl load was increased from 10 to 15 Gm. per day. This similarity in response suggests a symmetrical adaptation of several enzymes.

The similarity both in the magnitude of the adaptive increases of these five enzyme systems and in the magnitude of the net ammonia excretion after substrate loads suggests that when substrate is furnished in abundance by the administration of appropriate amino acids, these enzyme systems apparently can contribute equally to urinary ammonia. However, under normal circumstances there is a considerable variation in the concentration of precursor amino acids in plasma. In plasma, glutamine constitutes 20 to 25 per cent of the free α-amino nitrogen (15, 28) and *l*-alanine and glycine, another 24 per cent (29); *d*-alanine is apparently absent and asparagine is present in trivial concentrations (28). The contribu-

[6] The changes in enzyme activity when the chronic NH_4Cl load was increased from 5 Gm. per day to higher levels were not used because the net increase in ammonia excretion after substrate at this level was negligible or small compared to the control values. On the other hand, the net increases in ammonia excretion after substrate when the subjects were maintained on 10 and 15 Gm. per day were larger and therefore were considered to reflect more accurately enzymatic alterations.

tion to urinary ammonia of the enyme systems which are capable of adaptation may therefore vary widely because of substrate limitation.

The biological role of these enzymes, capable of adaptation, is not adequately defined. Significant activity of *l*-amino acid oxidase has been demonstrated *in vitro* in only the rat kidney (8). Despite its exceedingly low turnover number of 6, it nevertheless appears to be active *in vivo* in the human kidney and evidence from these studies and those in the rat (16) indicate that adaptation occurs. The disparity between the *in vivo* and *in vitro* evidence of its activity may exist because the ideal conditions for demonstrating *in vitro* activity have not been elucidated.

More engimatic is the *d*-amino acid oxidase, an ubiquitous enzyme, found in all mammalian kidneys tested and having a high (1,440) turnover number (11). The presence of a highly active enzyme without apparent substrate beclouds its physiologic role. Nonetheless, the administration of *d*-alanine to human subjects resulted in augmentation in ammonia excretion and the evidence presented indicating adaptive change during chronic acid loading bespeaks a physiologic role. Amino acid racemases, capable of interconverting *l* and *d* forms, have been found in microorganisms (11). Such a racemase may be present in mammalian kidney and undergo adaptive change, thereby shunting, as acid load requires, a portion of the *l*-amino acid pool into *d*-amino acid substrate for ammonia production.

In contrast to the apparent physiologic importance in the renal regulation of acid-base balance of those enzyme systems showing adaptive changes is the role of glutamic dehydrogenase, aspartic transaminase and proline oxidase for which no evidence of adaptation was found. Lotspeich and Pitts failed to find any augmentation of ammonia excretion after the infusion of glutamic acid in acidotic dogs (2). Neither proline oxidase nor aspartic transaminase directly increase ammonia production (Table I). Both, however, augment the synthesis of glutamic acid which may then be deaminated by glutamic dehydrogenase, thereby increasing ammonia production. The failure of proline or aspartic acid administration to augment ammonia excretion progressively may, therefore, indicate that either proline oxidase and aspartic transaminase do not adapt or/and that glutamic dehydrogenase does not adapt.

The inferential identification of the adaptation of a specific enzyme by the administration of its precursor amino acid implicitly assumes that the rise in ammonia excretion under such circumstances is the consequence of the effect of the administered precursor amino acid on the specific enzyme system for which it is substrate. It is conceivable, however, that the administered amino acid might be converted by a transamination reaction to another substrate that would then act upon another enzyme system which then would be responsible for the augmented ammonia excretion. This possibility seems unlikely. All transaminases including glutamine-α-keto acid and aspargine-α-keto acid transaminase are pyridoxal phosphate and pyridoxamine phosphate dependent (13, 28); yet in pyridoxine deficient rats with reduced transaminase activity (30) the renal response to acid loads is normal, ammonia excretion is high and glutaminase adaptation occurs (31). This suggests that the transaminase systems do not contribute significantly to the renal production of ammonia. Moreover, it is noteworthy that even though amino acids can be readily transaminated to glutamate and glutamate to other amino acids, nevertheless the administration of glutamate, aspartate and proline resulted in only a very small increase in ammonia excretion. In addition, the adaptation of glutaminase (16–19), glycine oxidase and amino acid oxidase (16) has been shown *in vitro* to follow the administration of strong acid loads. For these reasons it seems likely that the changes in ammonia excretion after the administration of a specific amino acid can be ascribed to its effect on the enzyme system for which it is substrate.

The increased urinary ammonia excretion that follows amino acid administration was invariably associated with an increase in total acid excretion [NH_3 plus ($TA-HCO_3$)]. These findings differ from those of Orloff and Berliner (25), who failed to find any consistent increase in total acid excretion following the infusion of *dl*-alanine into dogs. The discrepancy may be related either to species difference or to the fact that their experiments, conducted during acute acidosis, were different from the present studies which were performed during chronic acidosis.

The increased urinary pH that attends the increased ammonia excretion following substrate loads may be the result of either increased diffusion of ammonia from tubular cell to tubular urine thereby titrating tubular H^+ and permitting further H^+ for Na^+ exchange by maintaining an increased gradient from cell to urine, or it may be the result of an increased exchange of cellular ammonium ion for urinary sodium ion which otherwise would have exchanged for H^+ (32).

The relationship between urine pH and urinary ammonia that follows the chronic administration of strong acid loads of increasing magnitude contrasts sharply with the inverse relationship that prevails during acute acid loading (33, 34). This rise in urine pH with increases in chronic NH_4Cl intake could result from the enhanced diffusion of ammonia from tubular cell to tubular urine caused by the high concentration gradients produced by accelerated ammonia formation. It is also possible that with increased activity of the renal ammonia-producing enzyme systems, the exchange of NH_4^+ for Na^+ increases to some extent at the expense of H^+ for Na^+ exchange, resulting thereby in a rise in urine pH. Whatever explanation is valid, one consequence of renal enzymatic adaptation is the excretion of increased amounts of ammonia at any given urine pH (19, 35).

Evidence from the present experiments indicates that the superimposition of an acute alkalosis in a subject chronically maintained on 15 Gm. of NH_4Cl per day prevents the usual increase in ammonia excretion after a substrate load and results instead in a sharp decline in ammonia excretion (Figure 8). Data concerning the effects of alkalosis upon ammonia production are conflicting. Van Slyke and associates (1) reported that the diminution in urinary ammonia excretion which attends the changing from hydrochloric acid acidosis to bicarbonate alkalosis is associated with a decreased renal utilization of glutamine (1, 9) and a decreased renal production of ammonia. Other evidence (36) indicates unaltered ammonia production and decreased urinary ammonia excretion associated with an acute alkalosis.

SUMMARY

One hundred twenty-five experiments were performed on five subjects maintained on chronic acid loads of increasing magnitude. Under these conditions precursor amino acids were administered and the renal ammonia producing enzymes in the human kidney inferentially identified. By comparing the magnitude of response in ammonia excretion to a fixed amino acid load at several different levels of ammonium chloride administration, those enzymes which adapt were identified. Evidence was presented indicating that, in the human kidney, glutaminase, asparaginase, glycine oxidase, *l*-amino acid oxidase and *d*-amino acid oxidase adapt to chronic acid loads whereas glutamic dehydrogenase, proline oxidase and aspartic transaminase do not.

ACKNOWLEDGMENT

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MECHANISM OF POSTOPERATIVE LIMITATION IN SODIUM EXCRETION: THE ROLE OF EXTRACELLULAR FLUID VOLUME AND OF ADRENAL CORTICAL ACTIVITY [1]

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Decreased renal excretion of sodium is a well-known feature of the immediate postoperative state (1–3). The mechanisms responsible have, however, not been clearly defined. While increased adrenal cortical secretion such as is generally associated with major surgical procedures (4–9) has been causally implicated by many investigators (1, 3, 10–13), there is considerable evidence that the changes in sodium excretion are independent of variations in the supply of adrenal cortical hormones (14–18).

The extracellular fluid volume, or more precisely that portion of it which is physiologically "effective," has been shown to be a major determinant of renal sodium excretion in normal individuals (19–21). Therefore variation in this volume warrants consideration as a possible etiological factor in the decreased postoperative excretion of sodium. The observations that fluid sequestration occurs in and about the traumatized tissues both in animals and in man indeed suggests that a dislocation of the body fluids may occur in the surgical patient and result in a contraction of the effective extracellular fluid volume.

The present study was designed to investigate the effect of isotonic expansion of the extracellular fluid volume on the postoperative response to the administration of sodium chloride. The data indicate that the usually observed impairment in natriuretic response is corrected by isotonic extracellular fluid volume expansion. In addition, studies in a patient with Addison's disease suggest that decreased natriuresis postoperatively occurs independently of enhanced adrenal cortical hormone secretion.

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METHODS

Five young males without cardiovascular, renal, hepatic or endocrine disease were studied before and after orthopedic surgical procedures of sufficient extent to produce major surgical trauma (Table I). One patient (No. 3, A and B) was studied before and after two Moore arthroplasties performed five weeks apart. Orthopedic patients were chosen for study in order to avoid the complicating factors of major gastrointestinal losses of fluids and electrolytes and to allow continued oral intake in the postoperative period. Commencing on the fifth or sixth day before surgery and continuing through the third postoperative day the patients were provided a daily diet containing approximately 43 mEq. of sodium and in addition received nonenteric coated sodium chloride tablets four times daily so that the total sodium intake was 197 mEq. per day. On the day of surgery sufficient isotonic saline solution was given intravenously to maintain the total intake, including the sodium content of the citrated blood, at 197 mEq. Blood loss during surgery was replaced so that in no instance was significant hypotension observed. Oral intake of fluid and food was usually re-instituted by evening of the day of surgery. The daily intake of fluids was recorded in five studies and it was possible to weigh three patients daily. Urine collected over 24 hour periods was analyzed daily for sodium, potassium, chloride, total solute and creatinine by methods previously described from this laboratory (22). Pre- and postoperative 17-hydroxycorticosteroid excretion was measured by the Reddy method (23).

On the second, third or fourth day before surgery each patient was studied as follows: After the usual breakfast and two hours of "basal" observation in recumbency a challenging infusion of 2,000 ml. of 0.9 per cent saline was administered intravenously over a 90 minute period. Urine was generally collected at 30 minute intervals from the beginning of the "basal" period until at least three hours after the infusion was begun; thereafter urines were collected at varying intervals for a minimum of three additional hours. Venous blood was obtained at the start of the infusion, 45 minutes later, and at the end of the infusion. Urine and blood were analyzed for sodium, potassium, chloride, creatinine and total solute. In those studies in which inulin and para-aminohippurate clearances were determined, blood was drawn at least hourly for these determinations (24, 25).

All postoperative experiments were carried out on the second day after surgery. In three of the orthopedic patients (Nos. 1, 3A & 4) the postoperative study was identical with that described preoperatively, except that urines could not always be collected at precisely 30 minute intervals. In the other three studies (Nos. 2, 3B and 5) the challenging infusion of 2,000 ml. of 0.9 per cent saline was administered *only after* the extracellular fluid volume had been expanded to such an extent that the lowered postoperative "basal" rate of sodium excretion approximated the preoperative "basal" rate. This initial expansion was achieved after two hours of observation by infusing normal saline solution at a rate similar to that used in the preoperative tests. Urine specimens collected during this infusion were analyzed immediately for sodium. When the lowered rate of sodium excretion reached the preoperative "basal" level the challenging infusion of 2,000 ml. of saline was administered over a 90 minute period. The rest of the study was carried out in a manner identical to the preoperative experiments and to the other postoperative studies.

In addition to the five orthopedic patients, one patient (No. 6) with well-documented Addison's disease was studied before and after cholecystectomy. The relevant clinical data in this patient have been reported elsewhere in connection with other studies (26). This patient had been symptom-free while taking 50 mg. of cortisone orally each day and receiving monthly injections of desoxycorticosterone trimethylacetate (50 mg.). His last injection of the latter had been given 24 days prior to surgery and none was given subsequently until after the conclusion of the studies reported here. Just preceding the present study the patient had undergone other investigative procedures while receiving a daily diet containing less than 5 mEq. of sodium and an oral dose of 50 mg. of cortisone daily. He was prepared for surgery in the following manner: From the fifth preoperative day through the third postoperative day he

TABLE I

Clinical data

Patient no.	Operation (grade of trauma)*	Age	Anesthesia†	Postoperative medication		Volume of blood transfusion
				First day	Second day‡	
						ml.
1	Sub-trochanteric osteotomy and hip fusion (8)	25	Thiopental sodium Nitrous oxide Nisentil® Succinyl choline chloride	Morphine sulfate	Meperidine hydrochloride, 75 mg. (× 2)	3,000
2	Open reduction of tibial fracture with iliac bone grafts (6)	40	Pontocaine spinal	Meperidine hydrochloride	Codeine phosphate, 60 mg. (× 1)	0
3A	Left Moore arthroplasty (5)	36	Nupercaine spinal I.V. Nembutal®	Morphine sulfate	Codeine phosphate, 60 mg. (× 2)	300
3B	Right Moore arthroplasty (5)	36	Nupercaine spinal Promethazine	Morphine sulfate	Codeine phosphate, 30 mg. (× 2)	500
4	Posterior lumbar spine fusion (5)	46	Thiopental sodium Nitrous oxide Nisentil® Succinyl choline chloride	Morphine sulfate	Meperidine hydrochloride, 50 mg. (× 1) Codeine phosphate, 60 mg. (× 2)	1,000
5	Hemi-laminectomy L4–5 with disc removal (4–5)	22	Thiopental sodium Nitrous oxide Xylocaine®, local	Meperidine hydrochloride	Meperidine hydrochloride, 50 mg. (× 1) Codeine phosphate, 60 mg. (× 2)	0
6	Addison's disease: cholecystectomy and appendectomy (4–5)	41	Thiopental sodium Ether d-Tubocurarine Succinyl choline chloride	Meperidine hydrochloride	Codeine phosphate, 60 mg. (× 2)	0

* Grade of trauma based on a scale of 1–10 (1).

† Pre-anesthetic medication in all patients included: Nembutal® 100 mg.; either atropine 0.4 mg. or scopolamine 0.4 mg.; and morphine sulfate 10 mg., except in Patients 4 and 6 who received meperidine hydrochloride 100 mg. and 50 mg., respectively.

‡ Medication given for pain either during or within three hours after the challenging infusion of saline. The figures in parentheses represent the frequency of dosage during this period.

TABLE II

Daily balance data *

Patient no.	Day no. →	−5	−4	−3	−2	−1	0 Surgery	+1	+2	+3
	Intake Na	197	197	197	505†	197	287‡	197	505†	197
	Urine Na		82	226	414	300	110	100	162	144
1	Urine K		38	59	83	74	56	47	48	39
	Urine Cr		1,171	1,466	1,750	1,512	1,126	1,733	1,505	2,012
	Urine vol.		710	1,715	3,423	2,400	2,035	1,065	1,440	1,500
	Intake vol.		3,000	3,100	5,100	3,100	3,400	3,190	5,130	2,850
	Intake Na	197	197	523†	197	197	197	197	831†	197
	Urine Na	167	196	495	192	107	180	167	540	280
	Urine K	116	88	70	44	81	100	79	69	47
2	Urine Cr	2,217	2,074	2,520	1,157	1,209	1,980	2,066	2,000	1,841
	Urine vol.	2,026	1,920	4,925	1,860	1,780	978	3,680	5,970	2,500
	Weight		185	184.5	185	182.3	180		183	184
	17-OH steroids (urine)		4.7		3.0	6.2		18.4		5.2
	Intake Na	197	197	197	505†	197	197	197	505†	197
	Urine Na	186	174	196	469	284	182	148	230	171
	Urine K	52	50	51	63	66	39	43	52	22
3A	Urine Cr	1,690	1,646	1,602	1,777	1,650	1,593	1,880	1,836	1,790
	Urine vol.	2,600	2,180	2,370	5,453	3,300	1,125	1,730	4,077	2,450
	Intake vol.	4,100	4,100	4,100	6,100	4,100	4,100	3,700	6,100	4,100
	17-OH steroids (urine)			6.1		2.9		17.5		10.4
	Intake Na	197	197	505†	197	197	197	197	800†	197
	Urine Na	149	160	414	269	158	135	169	465	265
	Urine K	75	68	66	59	53	45	42	56	17
3B	Urine Cr	1,691	1,780	1,794	1,878	1,850	1,716	2,224	2,106	1,760
	Urine vol.	1,500	2,620	3,658	1,890	943	852	1,540	4,302	2,350
	Intake vol.	3,685	4,100	3,100	3,100	3,100	3,100	4,100	8,000	4,100
	17-OH steroids (urine)				8.1	7.3		13.2		
	Intake Na	197	197	197	532†	197	197	197	532†	197
	Urine Na	131	190	177	443	194	195§		237	295
	Urine K	35	39	40	63	43	105§		34	18
4	Urine Cr	1,622	1,534	1,576	1,772	1,339	3,093§		1,736	1,720
	Urine vol.	660	760	1,000	3,109	1,800	100	1,730	1,295	1,590
	Intake vol.	3,000	3,000	3,000	5,000	3,000	2,500	3,800	5,000	3,000
	Weight	168.8	164.5	168	169	168.5	166		167.8	170.5
	17-OH steroids (urine)		3.1	4.8			22§			4.8
	Intake Na	197	530†	197	197	197‖	197	197	705†	197
	Urine Na	140	366	247	152	123	156	110	526	208
	Urine K	35	66	83	71	48	63	35	34	19
5	Urine Cr	2,512	2,360	2,620	2,609	1,805	1,750	1,780	2,234	1,821
	Urine vol.	1,445	3,156	1,580	1,570	922	733	532	1,841	3,220
	Weight	151	151.3	151.2	150	147.5		148.5	147.6	147.5
	17-OH steroids (urine)			7.8	3.7			15.7		1.7
	Intake Na	197	197	197	530†	197	197	197	530†	197
	Urine Na	28	83	124	392	198	56	153	332	106
	Urine K	90	94	87	113	70	25	49	46	20
6	Urine Cr	1,485	1,645	1,484	1,703	1,345	780	2,145	1,663	1,409
	Urine vol.	910	2,720	2,560	4,193	3,230	413	1,805	3,316	1,695
	Intake vol.	4,000	4,000	4,000	6,000	4,000	3,280	3,000	6,000	4,000
	Weight	154.7	156.5	157.3	159	158	157.5		160	159

* Units of measurement: Sodium and potassium in mEq./day; creatinine in mg./day; volumes in ml./day; urinary 17-hydroxycorticosteroids in mg./day; weight in pounds.

† Day of challenging infusion of saline.

‡ Additional 90 mEq. of sodium was contained in the citrated blood.

§ Pooled two days' urine collection.

‖ Unknown electrolyte loss by emesis.

received two infusions daily, one in the morning and one at night, each containing 75 mg. of hydrocortisone administered over a six hour period for a total of 150 mg. daily. These infusions contained a total of 154 mEq. of sodium. An additional 43 mEq. of sodium was given orally, for a daily total of 197 mEq. The pre- and postoperative infusion studies were carried out in the manner outlined for the experiments in Patients 1, 3A and 4 above.

RESULTS

A. Orthopedic patients (Numbers one to five)

1. Postoperative daily excretion of sodium. On the day of surgery and for the three succeeding days all patients demonstrated some limitation of sodium excretion as evidenced by the fact that they excreted only 42 to 88 per cent of their sodium intake (Table II). Patient 1, who had the most extensive surgery, had the greatest degree of postoperative salt retention. On the day of surgery and the first postoperative day the decreased quantity of sodium excreted was almost always associated with an even greater reduction in the volume of water excreted, resulting in a more concentrated urine (Table II). By the second postoperative day there was, in general, slight reduction in the serum concentrations of sodium, chloride, total solute and creatinine, associated with a slight gain in body weight in those patients who could be weighed (Table III).

2. Natriuretic response to the challenging infusion of saline without prior expansion of the extracellular fluid volume. Postoperatively, all the patients had lower "basal" rates of sodium excretion than preoperatively (Table IV, Figures 1, 2, 3 and 4). All demonstrated increased natriuresis in response to the challenging infusions of saline. However, in two of the three patients (Nos. 1 and 4) the natriuretic response following surgery was only one-third as great as the preoperative response (Table IV, Figure 5).

3. Natriuretic response to the challenging infusion of saline with prior expansion of the extracellular fluid volume. Two thousand ml., 1,900 ml. and 1,300 ml. of 0.9 per cent saline were administered to Patients 2, 3B and 5, respectively, to raise the "basal" rate of sodium excretion in the postoperative study to the preoperative "basal" level. In each instance the challenging infusion, after prior expansion of the extracellular fluid volume, evoked a greater natriuresis postoperatively than preoperatively (Table IV, Figures 2, 4 and 5).

4. The renal excretion of total solute, chloride and potassium. For the most part, the excretion of total solute and chloride closely paralleled the excretion of sodium. Daily postoperative potassium excretion was generally somewhat lower than in the preoperative period. In most patients there was an increase in potassium excretion following the challenging infusions of sodium chloride in both the pre- and postoperative periods.

5. Changes in glomerular filtration rate (GFR) and renal plasma flow (RPF). In general, the clearances of creatinine, inulin and para-aminohippurate were higher in the postoperative "basal" periods than preoperatively (Table IV). The challenging infusion of saline did not result in any consistent change in GFR or RPF.

B. Patient No. 6, with Addison's disease, who re-received a constant dose of hydrocortisone and underwent cholecystectomy

The patient with Addison's disease had been taking a diet extremely low in sodium content (5 mEq. daily) for the week prior to the present study in conjunction with other investigations. During that time, while receiving 50 mg. of cortisone daily, he demonstrated a normal response to sodium restriction, reducing his urinary excretion to 8 to 10 mEq. daily. The abrupt change on the fifth preoperative day to a dietary intake of 197 mEq. of sodium daily and the administration of 150 mg. of hydrocortisone daily was associated with steady weight gain during the preoperative phase of this study, while daily sodium excretion increased progressively (Table II). After cholecystectomy sodium excretion decreased (Table II) and a lesser natriuretic response to the challenging infusion of saline was observed (Table IV). This patient had difficulty with complete bladder emptying on the day of receiving the challenging infusion postoperatively. Accordingly his sodium excretion is expressed in terms relative to creatinine excretion (Table IV).

DISCUSSION

The results of the present study confirm previous observations of sodium retention (1–3, 27–31) and decreased natriuretic response to salt ad-

TABLE III

Concentrations of the blood constituents on the days of the saline "test" infusions

Patient no.		Preoperative									Postoperative								
		Serum							Venous blood		Serum							Venous blood	
		Na	K	Cl	Solute	Cr	In*	PAH*	Hgb	Hct	Na	K	Cl	Solute	Cr	In*	PAH*	Hgb	Hct
		mEq./L.	*mEq./L.*	*mEq./L.*	*mOsm./L.*	*mg. %*	*mg. %*	*mg. %*	*Gm. %*	*%*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mOsm./L.*	*mg. %*	*mg. %*	*mg. %*	*Gm. %*	*%*
1	a.†	141	4.3	105	278	0.61			13.1	42	138	4.3	102	280	0.64			13.0	40
	b.‡	143	4.2	110	282	0.61			11.8	38	138	4.3	106	278	0.66			11.6	36
2	a.	137	4.6	104	276	0.82	10.7	1.28	14.0	48	135	4.2	101	266	0.71	8.8	1.17	13.1	45
	b.	139	4.3	109	277	0.74			12.7	47	140	4.1	110	279	0.64			11.4	37
3A	a.	139	3.8	106	281	0.69			14.0	42	130	3.9	97	264	0.65			11.7	38
	b.	141	4.0	112	283	0.62			12.5	38	133	4.1	105	266	0.64			9.9	31
3B	a.	140	3.9	105	284	0.79			15.5	46	132	3.7	100	269	0.71			12.1	36
	b.	140	4.1	111	285	0.75			13.1	39	135	3.8	110	273	0.65			10.0	29
4	a.	141	4.0	105	283	0.71	14.8	1.76	14.8	45	131	3.9	97	265	0.74	11.5	1.14	11.7	36
	b.	143	4.0	110	286	0.76			13.9	42	133	3.7	103	268	0.68			10.6	32
5	a.	139	3.9	104	274	0.76	14.4	1.29	16.4	49	133	3.6	98	264	0.62	8.9	0.73	15.5	46
	b.	141	3.9	109	278	0.66			15.4	47	134	3.7	104	267	0.64			13.5	42
6	a.	138	3.7	102	283	0.82	17.2	1.77	15.3	48	135	3.5	95	276	0.89	12.9	1.25	12.5	40
	b.	140	3.8	107	285	0.73			14.0	43	137	3.5	100	273	0.82			12.4	39

* Average values of inulin and para-amino hippurate when the serum levels were constant.
† a—Values immediately before the saline infusion.
‡ b—Values immediately after the saline infusion.

TABLE IV

Renal excretion of fluid and solutes during the experimental periods *

Patient no.	Preoperative									Postoperative								
	Time elapsed	Na	K	Cl	Solute	Flow	C_{Cr}	C_{In}	C_{PAH}	Time elapsed	Na	K	Cl	Solute	Flow	C_{Cr}	C_{In}	C_{PAH}
	min.	*μEq./min.*	*μEq./min.*	*μEq./min.*	*μOsm./min.*	*ml./min.*	*ml./min.*	*ml./min.*	*ml./min.*	*min.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*μOsm./min.*	*ml./min.*	*ml./min.*	*ml./min.*	*ml./min.*
1	− 60 to 0	281	89	311	1,200	2.1				−120 to 0	17	32	20	371	0.4	186		
	0 to 120	408	101	442	1,373	8.3	190			0 to 120	87	52	112	628	0.9	176		
	120 to 180	808	142	818	2,128	7.7	174			120 to 270	232	61	253	964	1.7	181		
	180 to 270	913	161	926	2,349	7.1	198											
2	− 60 to 0	163	60	89	938	4.2	167			− 80 to 0	105	47	100	830	10.0	213		
	0 to 90	445	103	413	1,471	7.0	175	147	720	0 to 90	266	73	288	1,112	7.1	194	172	900
	90 to 180	641	148	633	1,895	4.5	178	166	670	90 to 180†	750	103	798	2,137	10.0	208	177	840
	180 to 240	522	108	514	1,580	3.8	169	152	640	180 to 240	465	90	502	1,430	2.6	210	168	700
3A	− 60 to 0	146	44	180	586	2.6	164			− 60 to 0	50	35	83	498	0.7	230		
	0 to 90	234	74	242	795	7.5	176			0 to 90	74	38	149	660	3.9	230		
	90 to 150	557	103	613	1,385	5.7	178			90 to 150	276	69	382	1,075	2.8	220		
	150 to 210	481	77	542	1,265	3.0	178			150 to 210	422	72	511	1,365	2.6	226		
3B	−130 to 0	113	37	149	635	1.5	170			−120 to 0	38	30	70	822	1.8	240		
	0 to 120	292	104	358	1,139	4.7	179			0 to 120†	150	35	241	885	2.7	212		
	120 to 180	484	161	605	1,608	4.7	176			150 to 210	668	62	775	1,930	7.3	227		
	180 to 270	460	88	520	2,530	4.7	176			210 to 270	413	77	507	1,313	5.8	204		
	270 to 390	409	33	404	1,243	4.9	175			300 to 360	659	112	769	1,815	5.2	210		
4	− 60 to 0	169	35	118	785	1.5	194	141	607	−300 to 0	111	34		676	0.9	220		
	0 to 90	448	58	362	1,311	6.5	171	127	590	0 to 210	222	36		928	1.3	212	122	710
	90 to 210	605	82	578	1,368	4.4	159	127	570									
	210 to 600	449	45	495	1,060	2.0	168			210 to 580	253	27		770	1.2	203		
5	− 90 to 0	180	46	113	955	5.6	186	113	763	− 90 to 0	70	24	70	624	1.8	206	190	1,040
	0 to 90	477	78	438	1,523	4.7	175	127	750	0 to 90	248	36	280	1,041	2.0	240	214	1,368
	90 to 180	587	119	611	1,697	3.3	188	129	726	90 to 180†	599	50	631	1,728	4.5	240	190	1,300
	180 to 240	498	76	501	1,460	2.4				180 to 240	419	42	456	1,247	2.0			
	240 to 510	434	33	440	1,290	3.4				240 to 510	470	38	490	1,400	3.4			
6‡	− 90 to 0	111	87	57	600	2.0				−390 to 0	70	20	70	320	0.6			
	0 to 90	310	30	293	1,014	5.6	136	107	490	0 to 90	176	17	166	573	2.4			
	90 to 180	618	35	538	1,628	4.8				90 to 180	343	16	330	924	2.7	149	125	683
	180 to 390	448	35	419	1,124	3.9				180 to 330	309	21	307	834	1.8			

* Two thousand ml. of 0.9 per cent saline was infused from 0 to 90 minutes unless otherwise specified.

† The challenging infusion of 2,000 ml. of 0.9 per cent saline was administered after the postoperative "basal" rate of sodium excretion was raised to at least the preoperative "basal" level by the prior administration of 2,000 ml. of 0.9 per cent saline in Patient 2, 1,900 ml. in Patient 3B, and 1,300 ml. in Patient 5.

‡ All excretion rates for Patient 6 are expressed per mg. of excreted creatinine because of difficulty with complete bladder emptying. Clearance data are averaged for the entire period of testing.

ministration in the postoperative period (28, 32, 33). Renal excretion of sodium is determined both by the filtered load (GFR × serum sodium concentration) and the tubular reabsorption of sodium (34). In Patients 2 and 5, whose GFR was measured by inulin clearance, the calculated filtered load of sodium was actually greater in the postoperative period than preoperatively, despite a decreased serum sodium concentration after surgery. These data in conjunction with similar results in other patients in whom only creatinine clearance was determined, suggest that postopera-

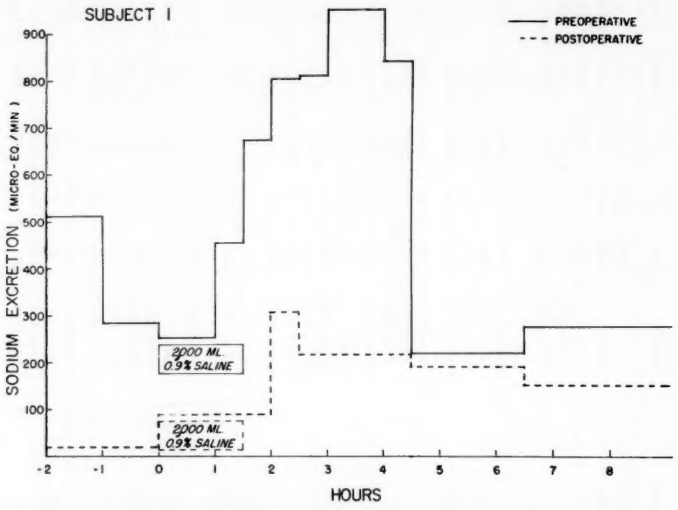

FIG. 1. PRE- AND POSTOPERATIVE NATRIURETIC RESPONSES TO THE CHALLENGING INFUSION OF SALINE IN PATIENT 1

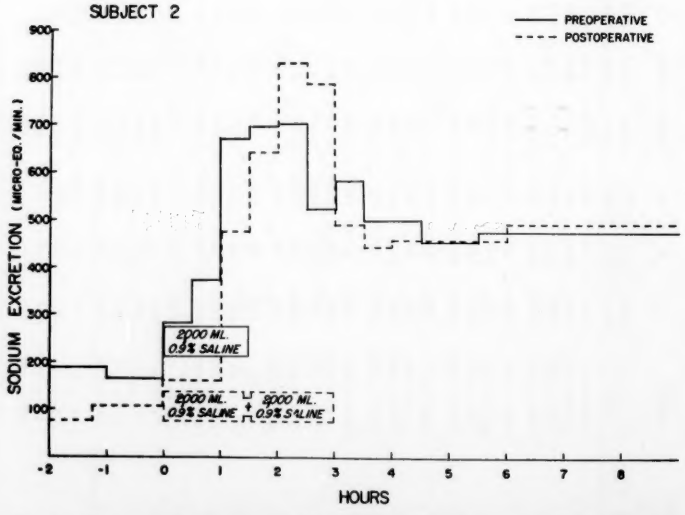

FIG. 2. PRE- AND POSTOPERATIVE NATRIURETIC RESPONSES TO THE CHALLENGING INFUSIONS OF SALINE IN PATIENT 2

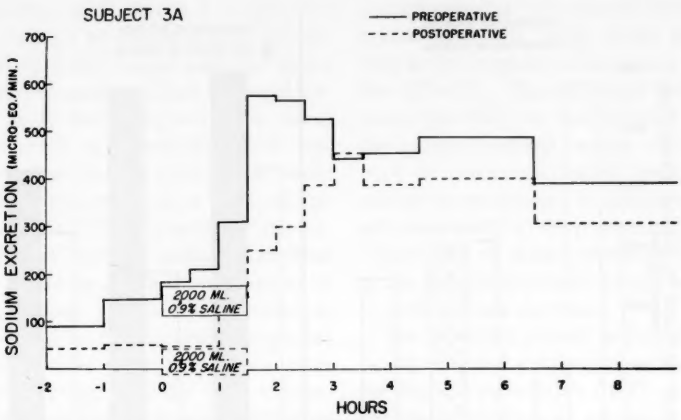

FIG. 3. PRE- AND POSTOPERATIVE NATRIURETIC RESPONSES TO THE CHALLENGING INFUSIONS OF SALINE IN PATIENT 3A

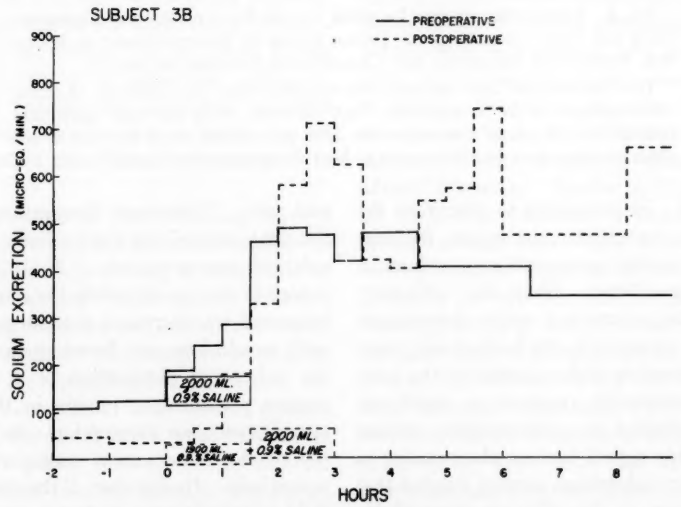

FIG. 4. PRE- AND POSTOPERATIVE NATRIURETIC RESPONSES TO THE CHALLENGING INFUSIONS OF SALINE IN PATIENT 3B

tive impairment in sodium excretion need not be related to decreased filtered load. Whether or not the decreased postoperative serum sodium concentration influences sodium excretion independent of filtered load remains unclear. While the precise mechanism of the observed postoperative increase in glomerular filtration rate noted in this and in other studies (35) is not known, it may be related to the reported influence of fever [3] upon glomerular filtration rate (34, 36).

In view of these observations, it seems reasonable to turn our attention to the possible role of enhanced renal tubular reabsorption of sodium in the impairment of sodium excretion in the post-

[3] Each patient had an oral temperature varying from 101 to 102° F. for one to three days following surgery.

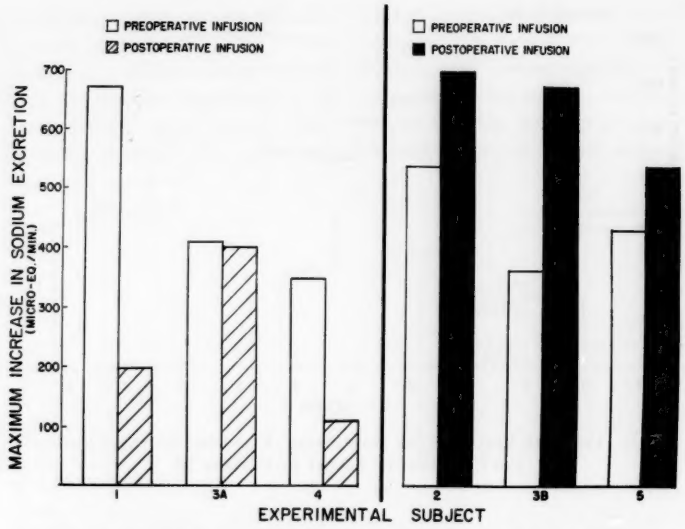

FIG. 5. COMPARISON OF THE INCREASE IN THE RATE OF SODIUM EXCRETION FROM THE PREINFUSION CONTROL PERIOD TO THE 60 MINUTE PERIOD OF MAXIMAL NATRIURESIS FOLLOWING THE CHALLENGING INFUSION OF SALINE

The cross-notched bars represent the response when only 2,000 ml. of saline was employed in the postoperative "test" infusion, while the solid black bars indicate the natriuretic response to the 2,000 ml. infusion when the rate of sodium excretion was *first* elevated to at least the preoperative "basal" level.

operative period. In attempting to determine the stimulus for any increased reabsorption, the normal stimuli for tubular conservation of sodium in man must be considered. Since the "effective" extracellular fluid volume is a major determinant of renal sodium excretion in the normal individual (19–21), a contraction of this portion of the body fluids might conceivably represent a significant physiological stimulus to postoperative sodium retention. In this regard various observations in animals and man undergoing surgery suggest that such a contraction in the effective extracellular fluid volume may indeed occur postoperatively. For example, sequestration of fluid and electrolytes occurs in and about traumatized tissues and surgical wounds (27, 37–45); fluid accumulates in the intestinal wall and lumen following manipulation during gastrointestinal tract surgery (38, 40, 41, 46–48), and in serous cavities (38) following thoracic and abdominal surgery. These collections of fluid may be as great as 4 to 5 per cent of body weight (37, 41, 42, 46), an amount probably sufficient to result in sodium retention in normal man. Numerous observations suggest that the *total* extracellular fluid volume may actually be enlarged postoperatively (13, 31, 32, 49–52), and indeed in the present study the inulin space, when measured, was increased after surgery.[4] However, such conclusions are based upon an increase in the volume of distribution of a variety of substances presumed to remain in the extracellular compartment, an assumption which may not apply to the same extent postoperatively as preoperatively. In any case, if the total extracellular fluid volume is expanded after surgery, this may well be the result of accumulation in the traumatized areas at the expense of the physiologically effective extracellular fluid volume (37, 41–44, 46). The operated patient may thus be likened to a patient with cirrhosis and ascites whose total extracellular fluid volume is expanded, but whose effective volume may well be contracted (53, 54).

While the mechanism of the decreased "basal" rate of sodium excretion postoperatively is not

[4] The volume of distribution of inulin was 16 to 38 per cent greater postoperatively than preoperatively.

clear, the possibility exists that it is a reflection of a contracted effective extracellular fluid volume. Indeed recent studies of Strauss, Lamdin, Smith and Bleifer have demonstrated that the renal excretion of sodium is altered by extremely small changes (1 per cent) in extracellular fluid volume (55). Consequently, the attempt was made to expand the effective extracellular fluid volume (in Patients 2, 3B and 5) with saline to the extent that the "basal" rate of sodium excretion postoperatively would be in the same range as in the preoperative values. Under these conditions the challenging infusion of saline evoked a greater natriuresis than in those instances without prior expansion of effective extracellular fluid volume. The fact that this natriuretic response following expansion of the extracellular fluid volume actually exceeded that of the preoperative response may be related to the fact that more saline was given prior to the challenging load than was actually required. This was in part due to the slight delay in determining the sodium content of the urine during which time the infusion of saline was continued, and to the fact that the peak natriuretic response to the expanding infusion had probably not yet been attained when the challenging infusion was begun.

If the sequestration of fluid following surgery accounts for the decreased natriuretic response to salt administration by producing a contraction of the effective extracellular fluid volume, it might be expected that restoration of this volume would prevent the diminished postoperative natriuretic response. On the other hand the fact that expansion of the extracellular fluid volume actually resulted in an increased natriuretic response in the postoperative period, does not constitute proof that the diminished rate of excretion was due to a contracted volume. It may well be that other stimuli for increased renal sodium reabsorption were in some way influenced, modified or overshadowed by extracellular fluid volume expansion. Nevertheless, in view of other observations, previously discussed, which suggest the possibility of decreased effective extracellular fluid volume postoperatively, the present studies may be considered consistent with the thesis that following major surgery, a contracted volume may play an important role in producing the observed limitation in sodium excretion. The manner in which such a volume stimulus is perceived and the pathways of mediation to the renal tubule remain unknown both in the surgical patient and in the normal subject (19–21). Regardless of the precise mechanisms involved, the data indicate that postoperative patients without hepatic, renal, cardiopulmonary or endocrine disease retain the ability to excrete large amounts of administered saline at a rate comparable to the preoperative period, if their "basal" rate of sodium excretion is first increased to the preoperative level by the administration of isotonic sodium chloride.

The increased adrenal activity (17-hydroxycorticosteroids and aldosterone) associated with major surgical procedures (4–13, 56) has been frequently implicated as causative of the sodium retention of the postoperative period (1, 3, 5, 10–13). There are important observations, however, which suggest that *increased* endogenous adrenal cortical activity is not essential to the postoperative abnormalities of sodium metabolism (14–18). In adrenalectomized animals and man, and in patients with Addison's disease, normal fluctuations in sodium excretion can occur in response to changes in volume (19, 26) or pathological conditions (57–59) despite a constant supply of adrenal hormones. Similarly, the response, insofar as sodium excretion is concerned, to standardized trauma in adrenalectomized animals receiving constant doses of adrenal cortical extract differs in no way from nonadrenalectomized animals experiencing similar trauma (14). Indeed, adrenalectomized animals kept alive with large salt intakes, but without adrenal cortical steroids, also show identical phenomena (18). In man, observations made immediately after bilateral adrenalectomy or after other surgical procedures in adrenalectomized patients have also revealed postoperative sodium retention (31, 60–62). This retention is of greatest significance in those studies in which hormonal dosage (and usually sodium intake as well) was kept constant (15–17). In the present study Patient No. 6, with Addison's disease, demonstrated marked and rapid renal sodium conservation while taking a diet low in sodium prior to the study reported here. In addition, both sodium retention and a diminished natriuretic response to saline administration were observed in this patient postoperatively, despite a constant supply of exogenous adrenal hormones

and the presumed absence of the endogenous secretion of either aldosterone or hydrocortisone. The possibility that surgery in some way altered the metabolic effects derived from the exogenous supply of hormone cannot, of course, be excluded, but it seems highly improbable that these effects would be enhanced.

While the increased plasma levels of 17-hydroxycorticosteroids, so frequently reported, on the day of surgery are perhaps in part due to decreased plasma clearance of these substances (63–66), it would be unlikely that diminished plasma clearance would account for significant plasma levels by the second postoperative day (67). Furthermore, the effect of hydrocorticosterone is not clearly that of producing increased tubular reabsorption of sodium, and in fact under certain circumstances there is evidence to suggest that such hormones may actually produce increased sodium excretion (68). It would appear that the increased aldosterone secretion associated with major surgical trauma (3, 69, 70) is not necessarily the prime mover in postoperative sodium retention, and almost certainly not in the adrenally insufficient subjects in whom sodium retention occurred despite all evidence indicating the complete lack of this hormone. Whether aldosterone plays any role in the production of altered sodium metabolism in the surgical subject with normal adrenal glands, remains unclear. Since a contraction of the volume has been shown to be a stimulus for aldosterone secretion (71), it is possible that if there is such a contraction postoperatively it may at least in part be responsible for the observed increase in urinary aldosterone. While both increased aldosterone secretion and decreased sodium excretion may be related to a contraction of the effective extracellular fluid volume, this does not imply any necessary causal relationship between these two effects.

The decreased potassium excretion that occurred following surgery in this study is in contrast to the observations reported by others (1–3). Since the intake of potassium was not strictly controlled, this decrease is probably accounted for by a reduced intake on the day of surgery and the first postoperative day.

The observation of decreased volumes of concentrated urine following surgery is similar to the observations reported by others (2, 3). This decreased excretion of water has been ascribed to increased antidiuretic hormone secretion (ADH) (3, 33, 72) which might well result from such factors as pain, emotion and a decreased effective extracellular fluid volume (21). While morphine and meperidine also result in decreased urine flow (73), this effect is probably independent of ADH secretion in man, as distinguished from its mechanism of action in animals (74, 75).

SUMMARY AND CONCLUSIONS

1. Postoperative and preoperative natriuretic responses to the intravenous administration of 2,000 ml. of 0.9 per cent saline were compared in five patients undergoing major orthopedic surgery. One patient was studied twice.

2. In all patients, the rate of sodium excretion prior to the infusions of saline was less in the postoperative experiments than preoperatively.

3. The diminished sodium excretion on the second postoperative day occurred despite a generally increased glomerular filtration rate, implying increased tubular reabsorption of sodium.

4. In two of three patients given the challenging infusion *without* prior expansion of the extracellular fluid volume postoperatively there was a strikingly smaller natriuretic response than preoperatively.

5. In each of three patients given the challenging infusion *with* prior isotonic expansion of the extracellular fluid volume postoperatively, the natriuretic response was at least as great as preoperatively.

6. One patient with Addison's disease, receiving a constant daily intake of hydrocortisone and saline, also demonstrated decreased sodium excretion and decreased natriuretic response to administered saline following major abdominal surgery.

7. These data are consistent with but do not prove the hypothesis that the postoperative limitation in sodium excretion, may to a significant extent, result from a contraction of the "effective" extracellular fluid volume.

8. Decreased postoperative sodium excretion, as well as the impaired natriuretic response to administered saline, apparently does not require enhanced adrenal cortical secretion.

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PANTOTHENIC ACID DEFICIENCY IN MAN[1]

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For the past seven years we have been studying the role of pantothenic acid in human nutrition and metabolism. This is part of a larger study aimed at defining more clearly the principles of clinical assessment of an essential nutrient in human subjects. It has been assumed that pantothenic acid is necessary for the maintenance of health in man but its abundance in natural foods was such that spontaneous deficiency either did not occur or had not been recognized. Presumably even in very poor diets, other vitamin deficiencies were limiting factors before pantothenic acid deficiency caused definite trouble. At least two avenues of investigation lay before us: to prepare and feed a diet devoid of pantothenic acid, or to give analogs of the vitamin and test possible antivitamin effects. Experiences in devising a deficient diet and testing several antagonists have been described (1–5). Clinical and laboratory abnormalities appeared in healthy young men given an artificial diet, partly synthetic and partly of purified ingredients, the whole virtually devoid of pantothenic acid. The mixture had to be given by stomach tube. The most extensive abnormalities occurred when in addition to the deficient diet large amounts of omega-methyl pantothenic acid were given.

By the time we began the work and subsequently, other investigators described in detail the nature of pantothenic acid deficiency in several laboratory animals (6–10). In growing animals, the earliest evidence of pantothenic acid deficiency is a decline in the rate of growth (11, 12). Strange variations occur in the color of the fur. Exudative lesions appear around the eyes and nose. They are pigmented and contain porphyrin (13). In swine a peculiar neuropathy causes queer stamping and a prancing gait resembling a goose step (14). The fertility of rats is impaired (15) and the fetal death rates of swine rise (16). Sudden death has been noted in several species, often precipitated by hemorrhagic destruction of the adrenal glands (17).

Coenzyme A, the active derivative of pantothenic acid, has functions which are thought to be disturbed and thus cause the metabolic changes observed in animals. Acetylation is retarded, as indicated by a decrease in the portion of para-aminobenzoic acid or sulfonamide excreted in the urine in the acetylated form. Adrenal cortical function is impaired as shown by depletion of sudanophilic and ketosteroid substances from the adrenals of rats, disappearance of the eosinopenic response to adrenocorticotrophic hormone (ACTH), fall in the blood level of glucose accompanied by increased sensitivity to insulin, and defective regeneration of adrenal cholesterol in rats forced to swim for a long time in cold water (18–21).

We designed our tests hoping to detect any abnormalities that might occur at an early and presumably reversible stage. Progress in finding regular abnormalities was slow. Some of the early results were not obtained in later studies. However some clinical and chemical abnormalities were consistent. Fatigue, apathy and malaise characterized the induced illness, with gastrointestinal disturbances common and personality changes and emotional disorders usual. Less regular were signs of cardiovascular instability such as tachycardia and lability of the arterial blood pressure with a tendency to orthostatic hypotension. Several subjects developed paresthesias, burning sensations of the hands and feet, and muscle weakness. In some tests infections were common, in others they were not. Biochemical abnormalities included an inconstant reduction in the degree of acetylation of para-aminobenzoic acid excreted in the urine, a reduction of urinary 17-ketosteroids, a loss of the eosinopenic response to ACTH.

[1] The major support of the Metabolism Section has been borne by a grant from the National Institutes of Health, Bethesda, with important assistance from the National Vitamin Foundation, The Nutrition Foundation, The Department of Medicine Trust Fund, Eli Lilly & Co., and Burroughs Wellcome, Inc.

abnormal glucose tolerance, and increased sensitivity to insulin. Water absorption and elimination were retarded as indicated by the Robinson-Power-Kepler test. Secretion of gastric hydrochloric acid and pepsin was curtailed drastically. Serum cholesterol fell quickly soon after the synthetic diet was started. Hypokalemia developed, accompanied in some instances by electrocardiographic changes. One subject who had many infections had a decrease in gamma globulins but in other subjects they were normal. The results were not completely consistent from test to test, and prompt and complete recovery did not always follow pantothenic acid administration.

We were uncertain whether unrecognized variations in the composition of the diet, the activity of the antagonist, or the inherent variability of the subjects was responsible. Were consistent abnormalities actually manifestations of pantothenic acid deficiency alone or was the diet inadequate in other respects? Did the liquid diet given by stomach tube alter the rate of absorption and utilization of nutrients? Another possible factor was a psychic one. What was the effect of being fed by stomach tube for many weeks? We thought this might alter metabolism as well as mood. Still another possibility was that omega-methyl pantothenic acid might exert some toxic or pharmacologic activity hitherto unrecognized and not related to the function of pantothenic acid. Also, some of the test procedures such as the daily administration of para-aminobenzoic acid or the weekly injection of ACTH might introduce artifacts. Was there an appreciable variability of different lots of omega-methyl pantothenic acid? These were the questions we had in mind in designing the tests herein reported.

THE DESIGN OF THE TEST

We selected six healthy men with the following characteristics:

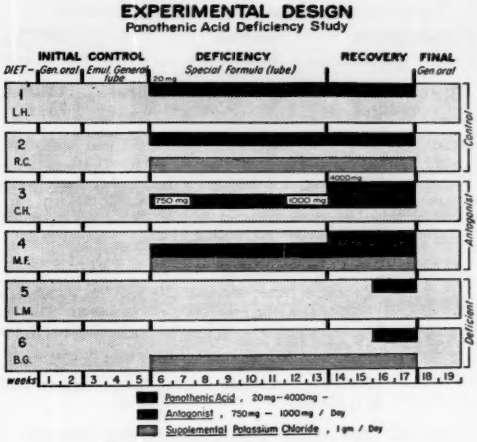

FIG. 1. THE PLAN OF THE STUDY

Each of the larger horizontal bars represents one subject. The diet is indicated at the top and the special regimens are indicated for each pair.

The subjects were paired by weight. During the tests none was aware that his regimen differed in any way from that of the other five subjects. Whenever a pill, capsule or injection was given to one pair or one subject, identical placebos were given to the others. Any procedures done to one was done to all. The general outline of such tests, the material and methods were the same as those in our earlier studies (1–5) except where a difference is noted specifically. Sampling and test procedures were the same for all subjects alike. During the first three weeks of the study, tube feeding of a general hospital diet, emulsified in a blender, was given to detect any psychic or physical effect of this procedure itself (Figure 1). Then we gave a formula very similar to that used in our previous studies (Figure 2). There were differences in the amount of the various vitamins we gave. We gave 75 per cent of the recommended daily allowances for each vitamin, suspecting that any excesses might mask the effect of a deficiency of pantothenic acid alone. The mineral supplements were changed in one important detail. Previously we had digested the casein with pancreatin in order to get it to go through the gastric tube. The resulting acid mixture

Patient	Age	Height	Weight	Previous health	Physical exam.	B.P.	Group
L. M.	29	73″	180	Cholelithiasis 1954	RUQ abd. scar	125/70	Deficient
B. G.	35	68″	174	"Rheumatism" 1943	Mild obesity	140/75	Deficient
C. H.	26	67″	145	Excellent	Normal	110/70	Antagonist
M. F.	21	70″	135	Excellent	Burn scar rt. abd.	120/70	Antagonist
L. H.	29	71″	170	Excellent	Normal	135/75	Control
R. C.	19	70″	165	Excellent	Dental caries	120/60	Control

	Basic Formula	
Carbohydrates	Sucrose	290 Gm.
	Cornstarch	75 Gm.
Protein	Casein (purified)	125 Gm.
	+ L. Cystine	750 mg.
Fats	Corn Oil	90 Gm.

Vitamins—Thiamine, 1.2 mg.; riboflavin, 1.5 mg.; pyridoxine, 210 mg.; ascorbic acid, 50 mg.; niacin, 6.0 mg.; vitamin D, 500 U.; vitamin A, 5,000 U.; vitamin B-12, 12 μg.

Minerals—Calcium biphosphate, 136 mg.; calcium lactate, 326 mg.; ferric citrate, 30 Gm.; magnesium sulfate, 138 mg.; dibasic potassium phosphate, 240 mg.; sodium biphosphate, 88 mg.; sodium chloride, 4.25 Gm.

(Potassium chloride 1 Gm. daily given to half of patients)

FIG. 2. THE "DIET" GIVEN TO ALL SIX MEN BY STOMACH TUBE

had a foul odor and was very disagreeable even when taken by stomach tube. We had given part of the sodium as sodium bicarbonate to neutralize the formula. In the present study, a pressure system was devised to force the mixture through the tube. This obviated the need for pancreatin and sodium bicarbonate. The sodium requirement was met by giving sodium chloride.

The experimental design paired the six men in three groups. One pair, L. M. and B. G., received the basic diet devoid of pantothenic acid. Nothing else was given except for the other essential vitamins and minerals, until the end of the "deficient period." At this time pantothenic acid was given in a dose of 4,000 mg. daily.

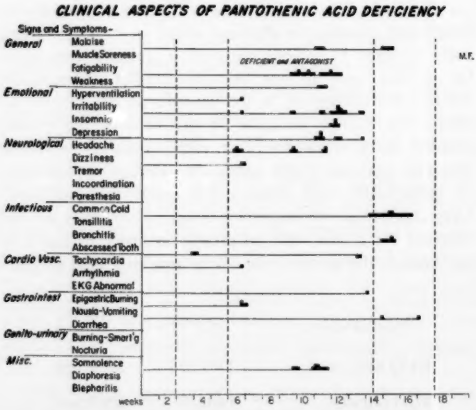

FIG. 3. SYMPTOMS IN M. F. (ANTAGONIST GROUP)

The quantitative aspect of all symptoms is indicated at three levels of mild, moderate and severe by using one, two or three blocks on the vertical axis for each day.

These subjects will be referred to as the "deficient pair."

Another pair, C. H. and M. F., received the same formula devoid of pantothenic acid but, in addition, they got 750 mg. of omega-methyl pantothenic acid. They are referred to hereafter as the "antagonist pair." Later the dose was increased to 1,000 mg. daily because some of the signs and symptoms which they had developed began to diminish spontaneously. At the end of the "deficient period" the antagonist was continued while 4,000 mg. of pantothenic acid daily was added. In the final week of the "recovery period" these two men also received an injection of 3.5 mg. of coenzyme A twice daily.

The remaining pair, L. H. and R. C., served as controls, receiving the basic formula supplemented by 20 mg. of pantothenic acid daily, together with all other essential vitamins and minerals given to the other subjects. These men will be referred to hereafter as the "controls" or the "control pair."

Because of the evidences of hypokalemia encountered in our previous studies, *one man in each pair* got a supplement of 10.8 mEq. of K daily.

Formerly we had found that 10 weeks was the longest period that the subjects could be kept on the experimental regimen of tube feeding. In this study it was possible to tube feed them for 15 weeks, largely because of very close personal care of the subjects, by having better recreational activities, and by the devoted cooperation of the nurses and attendants on the metabolic ward, not to mention the willingness of the subjects to continue a very difficult program.

Methods. The procedures employed to detect biochemical and clinical changes are as follows:

Each subject received his "food" by stomach tube at 11:30 a.m. and at 5:30 p.m. so that special tests and procedures did not delay the two daily meals. Each subject had six cups of black coffee, two bottles of Coca Cola®, and 100 Gm. of hard candy daily. The total intake of calories was 3,200. Each Monday, routine blood counts and urinalyses were done. On Tuesday, blood was obtained for various determinations and a glucose tolerance was done by the fingertip technique (22). On Wednesday, a "Thorn test" was done and 25 units of ACTH was administered (23, 24). On Thursday, gastric analyses were done, (25) and on Friday, an insulin tolerance test followed the withdrawal of blood for routine determinations. On alternate Saturdays a Robinson-Power-Kepler test (26) was performed, and on Sunday, para-aminobenzoic acid was administered in a single 500 mg. oral dose to measure the amount excreted in the urine in the acetylated form (27). Complete urine and stool collections were made daily, using appropriate methods for preservation. Thyroid function studies employed the protein bound iodine (28) and the 24 hour uptake of radioactive iodine (29). Urinary sodium and potassium were determined in a flame photometer but fecal analyses were not done. Seventeen ketosteroids were determined by a modification of the Zimmerman reaction (30). Determinations of nitrogen, cholesterol, total lipids

and phospholipids were done by standard methods (31–33). Serum protein electrophoresis was done by the filter paper method in a Durrum cell apparatus. Samples of the formula were preserved for analysis. A standard graded amount of recreation and exercise was available but the subjects were not required to participate.

RESULTS

Clinical observations

During the three week period of feeding the emulsified general diet, all six men remained in good health except for a few very minor troubles such as a toothache, an occasional headache, an episode of tachycardia and of mild tonsillitis. During the deficient period, as time progressed the antagonist pair developed serious personality

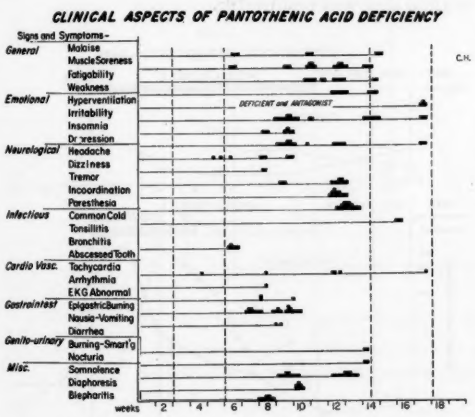

FIG. 4. SYMPTOMS IN C. H. (ANTAGONIST GROUP)

changes with irritability, restlessness, quarrelsomeness; and alternate periods of somnolence and insomia (Figure 3, 4). They began to complain of excessive fatigue after their daily walk. They would break out in a profuse sweat after trifling provocation or none at all. A little later the two men in the deficient group began to note similar complaints (Figure 5, 6). From this time on the condition of the deficient and the antagonist subjects became indistinguishable. All four men had a staggering gait and showed deterioration of their skill at pingpong. Frequently they refused to go for their daily walks, preferring to lie in bed all day. Gastrointestinal complaints became common, varying from epigastric burning to occasional regurgitation of small amounts of formula as they

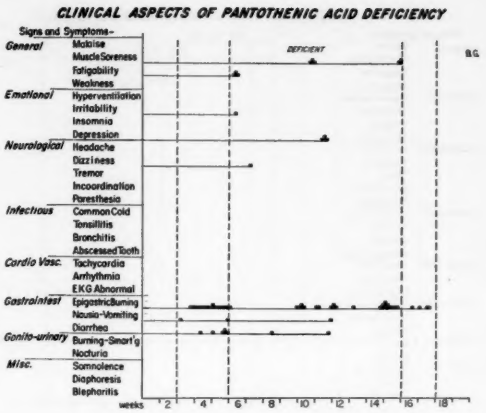

FIG. 5. SYMPTOMS IN B. G. (DEFICIENT GROUP)

withdrew the tube. Loud abdominal rumblings occurred frequently, sometimes accompanied by abdominal cramping and occasionally by diarrhea.

One subject developed paresthesias and "burning" of the soles of his feet which lasted only a few days and subsided spontaneously during the same phase of the test. Numbness of the hands, most distressing in the morning before arising, was fairly frequent in the two subjects receiving the antagonist.

Physical examinations revealed few objective findings other than transient increase of the tendon reflexes and faulty coordination associated with tremor.

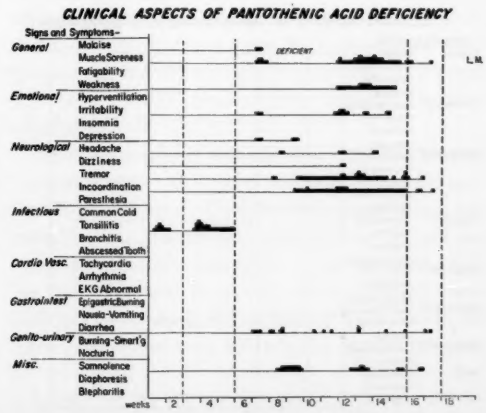

FIG. 6. SYMPTOMS IN L. M. (DEFICIENT GROUP)

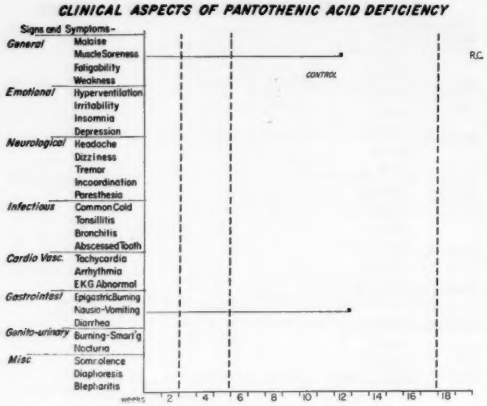

FIG. 7. SYMPTOMS IN R. C. (CONTROL)

The two controls remained well with no more complaints than we have found in any subject constrained in a metabolic ward (Figures 7, 8). The four deficient and antagonist subjects had few cardiovascular symptoms, although one (C. H.) had an arrhythemia which subsided spontaneously before we could get an electrocardiographic record of it. All the men maintained their normal weight throughout the study.

Laboratory results

Routine electrocardiograms remained normal except in one man who developed the T wave changes consistent with hypokalemia. He was getting supplemental potassium chloride and his serum level of potassium was consistently normal.

Blood counts showed a slight decline in hemoglobin, hematocrit and red cell counts resulting from the loss of blood withdrawn for testing. There was no difference in the degree of this mild anemia between the controls and the antagonist or deficient subjects. Urinalysis remained normal.

The erythrocyte sedimentation rate remained normal in the control pair, increased moderately in the deficient pair, but increased more in the antagonist pair (Figure 9). In one of the latter this increased sedimentation rate began to return to normal when large amounts of pantothenic acid were given. In the other, the rate became even faster, returning to normal only after the normal diet was restored.

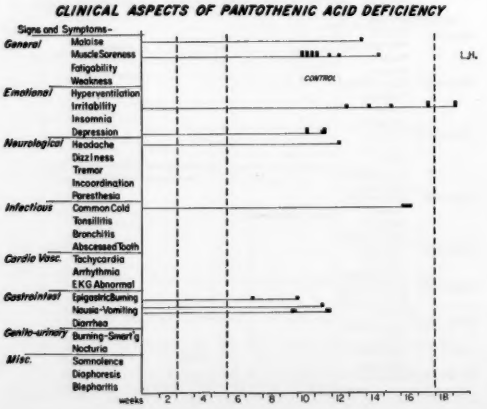

FIG. 8. SYMPTOMS IN L. H. (CONTROL)

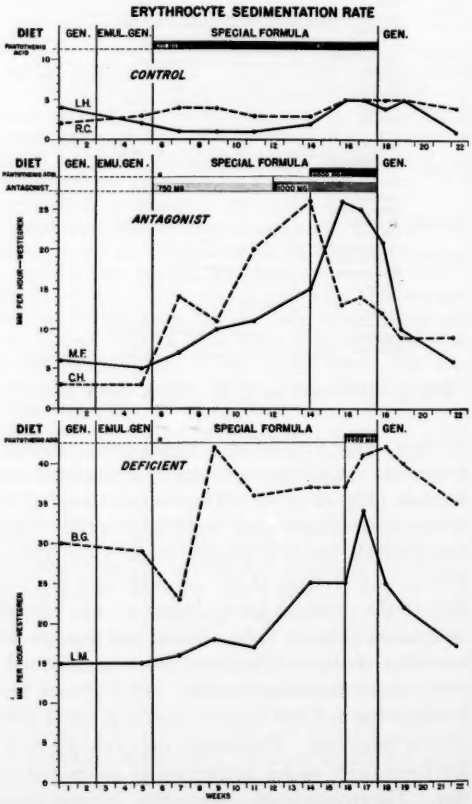

FIG. 9. ERYTHROCYTE SEDIMENTATION RATE IN THE CONTROL, ANTAGONIST AND DEFICIENT GROUP, RESPECTIVELY

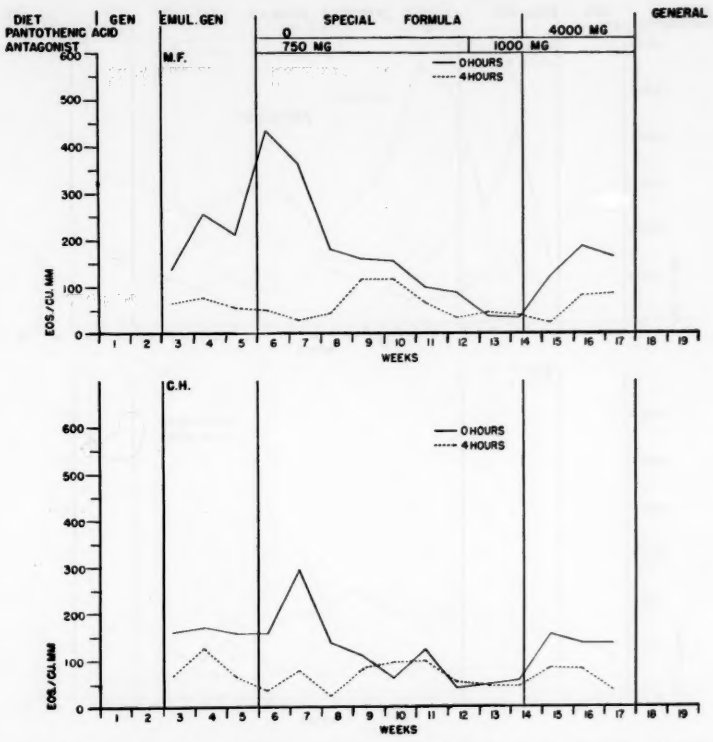

FIG. 10. THE EOSINOPENIC RESPONSE TO 25 MG. ACTH IN THE ANTAGONIST GROUP

The "zero hour" counts are joined by a solid line, while the "four hour" counts are jointed by a dotted line. A normal response is a fall of 50 per cent or more at four hours.

The eosinopenic response to ACTH disappeared in the antagonist pair (Figure 10) and in one of the deficient pair (Figure 11), while remaining normal in the controls (Figure 12). The initial eosinophil counts tended to fall, suggesting that a stress situation existed.

Serum levels of cholesterol and cholesterol esters began to fall soon after corn oil replaced the fats of the general diet. The degree of decline in cholesterol was identical in all three groups. The data are combined in Figure 13. Total lipids and phospholipids did not change during the experiment proper but when the men resumed eating a normal general diet, total lipids increased to twice the control values and there was a slight, consistent rise in phospholipids (Figure 14).

Levels of the serum protein bound iodine and the thyroidal uptake of radioactive iodine rose in all patients despite the fact that all subjects remained euthyroid and none had any clinical suggestion of hyper- or hypothyroidism[2] (Figures 15, 16). These observations will be reported separately.

Liver function, as measured by bromosulphalein clearance tests and serum proteins, remained normal in all subjects. Carbohydrate metabolism was variable, but glucose tolerance curves did not become significantly abnormal. The pattern which we had observed previously in pantothenic acid deficiency—a rapid rise followed by a sharp decline—developed only sporadically in two subjects.

[2] Assay of the formula for iodine revealed approximately 100 mEq. a day. This agrees with the urinary excretion of 86 to 112 mEq. per day by these men.

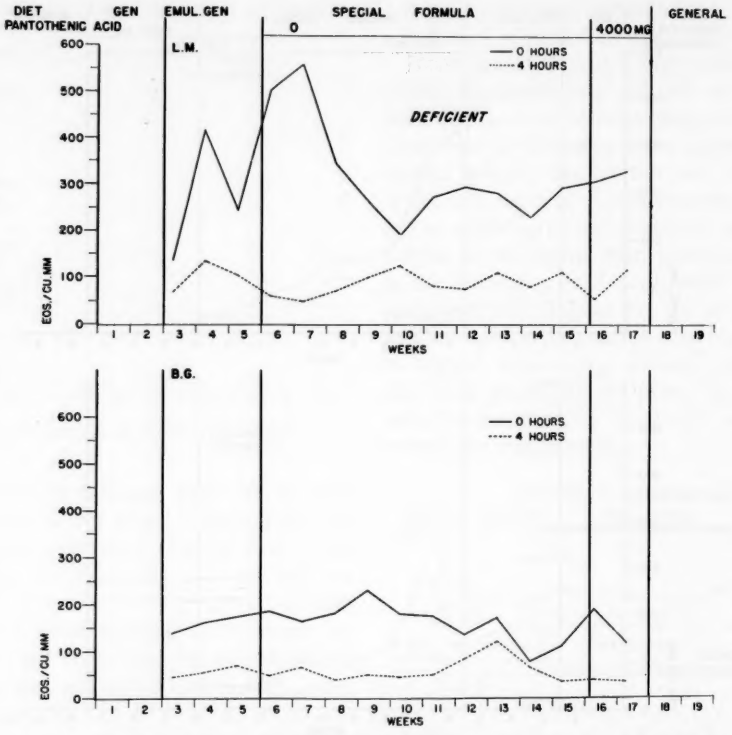

FIG. 11. THE EOSINOPENIC RESPONSE TO 25 MG. ACTH IN THE DEFICIENT GROUP

However, the sensitivity to insulin increased. The usual response to 0.1 units of insulin per Kg. of body weight was a fall of approximately 15 mg. per cent in blood sugar. One antagonist subject (C. H.) developed a 40 mg. per cent decline in blood sugar and complained of symptoms suggesting hypoglycemia (Figure 17). This was corrected by giving pantothenic acid.

The glycogenolytic response to adrenalin was distinctly abnormal in one of the antagonist pair (M. F.). That the drug was effectively absorbed was apparent from his development of tachycardia and slight tremor. In a later test, however, he had a normal rise in blood sugar.

Serum and urinary concentrations of sodium remained normal. For three subjects the formula supplied 20.5 mEq. of potassium daily. This approaches the minimal daily requirement. The other three men received an additional 1 Gm. of KCl daily (10.8 mEq. as K or a total of 31.3 mEq. K). The serum levels of potassium in those without the supplement fell to low normal levels. Those getting the supplement remained slightly higher. Urinary excretion of potassium approximated 10 mEq. per day in the unsupplemented and 20 mEq. per day in the supplemented group (Figure 18). Potassium balance studies were not done. Electrocardiographic tracings did not give evidence of hypokalemia except in the subject mentioned.

Studies of the degree of acetylation of para-aminobenzoic acid (PABA) in the urine failed to show any abnormalities. Similarly the excretion of 17-ketosteroids remained normal. This is in contrast to our previous experiments where the 17-ketosteroid excretion fell markedly.

Studies of the response to water diuresis were too variable to interpret because of a gastro-colic reflex resulting in prompt diarrhea in five subjects.

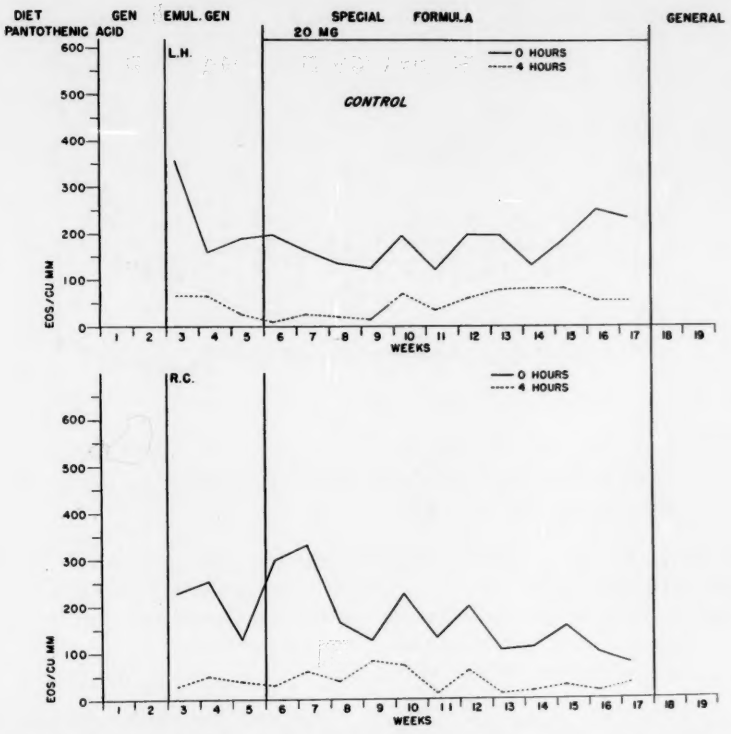

FIG. 12. THE EOSINOPENIC RESPONSE TO 25 MG. ACTH IN THE CONTROL GROUP

Pantothenic acid excretion in the urine was measured by a microbiological technique (34). In the antagonist pair, the results were unsatisfactory because of the inhibition of bacterial growth induced by the urinary excretion of omega-methyl pantothenic acid. While the general diet was being fed to the deficient pair, excretion averaged 4 mg. per day (Figure 19). When the fomula was started, this value declined gradually to less than 1 mg. per day. At the close of this period, when 4,000 mg. of calcium pantothenate was given daily, the urinary excretion rose to approximately 1,000 mg. daily. In the two men who received 20 mg. of pantothenic acid daily, the urinary excretion was about 18 mg. per day (Figure 20).

Nitrogen balance was measured throughout the study in all subjects. There was no striking degree of negative balance in any subject. In the antagonist pair the balance became positive by 2 to 4 Gm. per day after they were given pantothenic acid. When a general diet was resumed this posi-

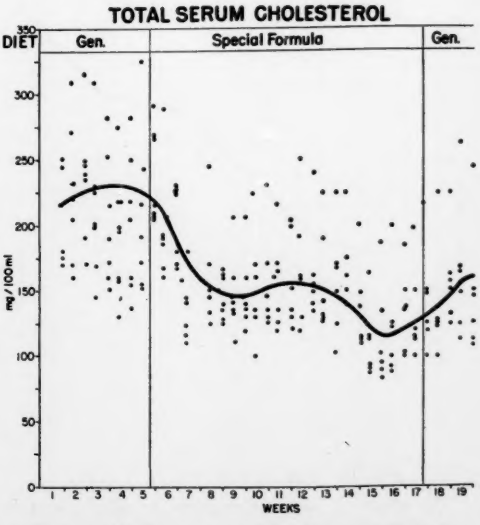

FIG. 13. CHOLESTEROL VALUES OF ALL SIX MEN
There was no difference among the three groups.

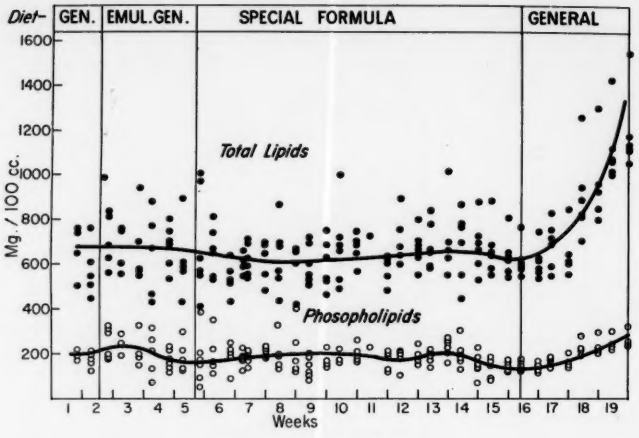

FIG. 14. SERUM FATS

Total fats in all six men remained normal until a general diet was resumed, whereupon the values rose.

tive balance increased further to 7 Gm. per day before decreasing (Figures 21, 22).

Studies of the serum proteins revealed minor and transient decreases of the gamma globulin concentration. The cause for the elevation of the erythrocyte sedimentation rate was not apparent from electrophoretic studies of serum. The gastric secretion decreased as before, but transiently, and to a lesser degree.

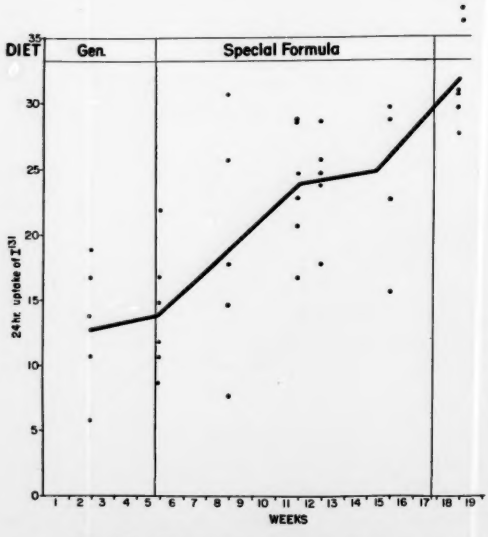

FIG. 15. RADIOACTIVE IODINE UPTAKE

The uptake of radioactive iodine in all six men increased steadily, but did not reach levels found in thyrotoxicosis.

DISCUSSION

This study has clarified some of the aspects of pantothenic acid deficiency induced in man under stringent experimental conditions. With the deficient diet alone, certain changes readily produced by a combination of a deficient diet and the antagonist, omega-methyl pantothenic acid, occurred. The changes were not as early or severe. Omega-methyl pantothenic acid hastened the development and increased the severity of the abnormalities. The effect was quantitative, not qualitative. We have noted that some lots of the antagonist seem to have less antivitamin effect than others. Indeed, one lot seemed to act as pantothenic acid itself. It is possible that the vitamin supplement varied in degree of purity. For these reasons we have found it necessary to employ the bioassay technique to evaluate every substance administered to the subjects. We plan to see whether large doses of omega-methyl pantothenic acid can produce the same abnormal state in subjects eating a normal diet.

The clinical picture of experimental pantothenic acid deficiency includes personality changes, fatigue, malaise, sleep disturbances and such neuro-

logical manifestations as numbness, paresthesias and muscle cramps. Impaired motor coordination also occurs, and may be accompanied by a peculiar gait. Gastrointestinal complaints include nausea, abdominal cramps, occasional vomiting and an increase in the passage of flatus. Epigastric burning sensations are common. Fatigue and headache usually accompany the sensation of weakness; this triad of symptoms is the most constant, persistent and annoying of all. Administration of pantothenic acid was followed by improvement of the paresthesias and muscle weakness, but fatigue and some degree of irritability persisted.

Of the laboratory tests which changed during the deficiency period, the loss of eosinopenic response to ACTH was the most consistent. One possible interpretation of this could be that the "stress" of the deficiency resulted in a maximal degree of eosinopenia (Figure 10) before ACTH was given. If adrenal cortical function had been impaired, other abnormalities should have occurred. Actually only one did occur—an increased sensitivity to insulin. With normal urinary 17-ketosteroids, normal glucose tolerance curves, and normal levels of sodium in blood and urine, we cannot invoke adrenal cortical hypofunction as the explanation.

Potassium requirements in man are taken care of in natural diets. Analysis of the average normal diets reveals that an adult ingests from 50 to 100 mEq. of potassium a day (35). Normally 35 to 90 mEq. are excreted in the urine and 5 to 10 mEq. are eliminated in the feces as unabsorbed or excreted potassium (36). The amounts lost in sweat normally are very small (37). Normal

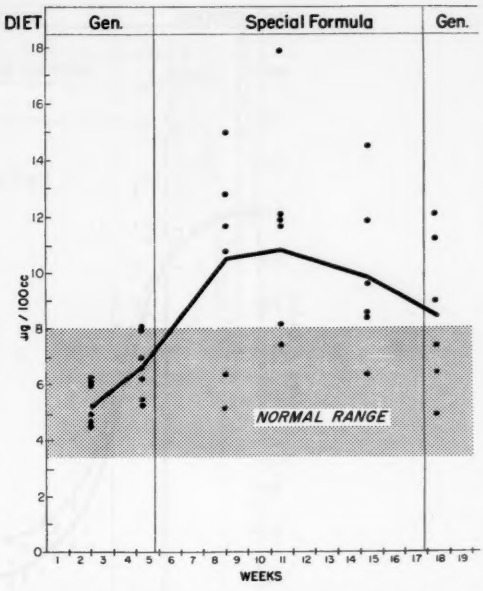

FIG. 16. SERUM LEVEL OF PROTEIN BOUND IODINE
The serum PBI rose to "thyrotoxic" levels in all six men, then began to decline.

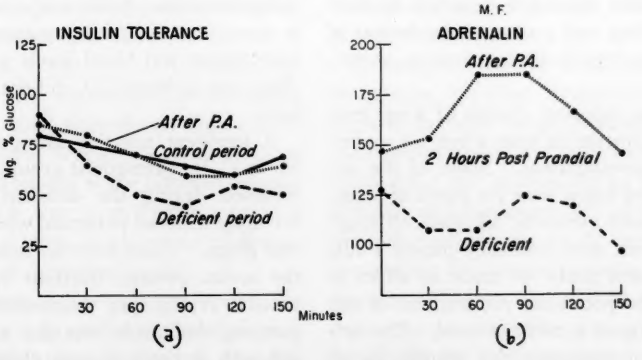

FIG. 17. CARBOHYDRATE METABOLISM
The response to insulin in M. F. (antagonist group) became exaggerated (a). The glycogenolytic response to epinephrine was impaired in M. F. (antagonist group) (b). These abnormalities were corrected by pantothenic acid.

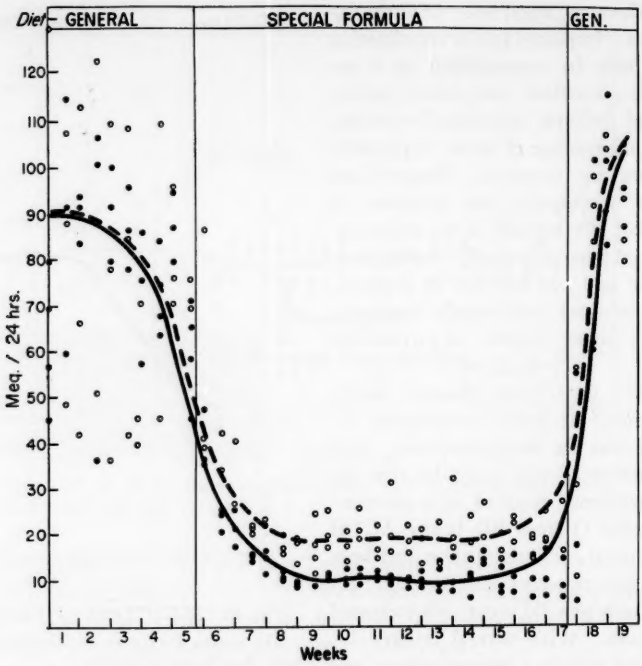

FIG. 18. URINARY EXCRETION OF POTASSIUM

Potassium excretion fell rapidly in all six men and became lower in the men not receiving the extra potassium (solid line). Those with the added 1 Gm. of KCl to the diet had a doubled excretion of K.

kidneys are not nearly so efficient in conserving potassium as sodium (38). Much is known about potassium and other electrolyte depletion in diarrhea, (39) vomiting and prolonged intubation of the gut as well as certain observations in starvation (40).

There were no reported studies of long term potassium requirements in man when we undertook our early investigations. Some of the abnormalities we now know were the result of potassium deficiency and metabolic alkalosis although perhaps pantothenic acid deficiency played a role too. In the present study we made an effort to gather data on the potassium requirement of our subjects by aiming at a minimal level. The urinary excretion of potassium fell rapidly in all subjects when the experimental formula was used. It stabilized at a lower level, ranging from 10 to 20 mEq. a day. On many occasions the serum potassium level of those getting only 20.5 mEq. daily fell to what we accept as the normal lower limit, but they developed no symptoms suggestive of hypokalemia. In the subjects with supplements of potassium the urinary potassium levels were a little higher and blood levels were normal. The slight rise in blood CO_2 did not eventuate in alkalosis.

A transient negative nitrogen balance did occur in C. H. (antagonist group). It was partially reversed during the deficient period and was promptly restored to normal when pantothenic acid was given. There were no substantial changes in the serum protein fractions to explain the increased erythrocyte sedimentation rate. Hypogammaglobulinemia was not a constant finding, although at times gamma globulin levels did decline. The incidence of infections in this experiment was not unusual and was about the same among the three test groups.

The administration of massive doses of panto-

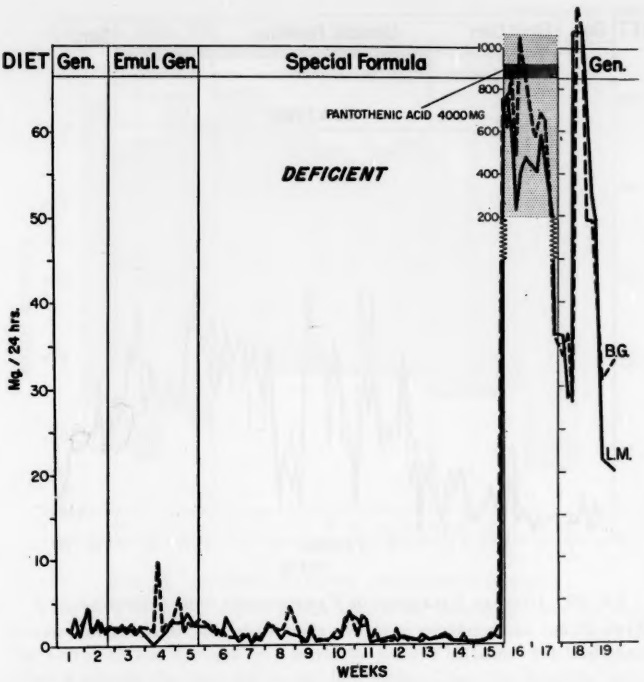

FIG. 19. URINARY EXCRETION OF PANTOTHENIC ACID (DEFICIENT)

Pantothenic acid excretion approached zero after the 11th week of the deficient diet. The men excreted only about ¼th of the 4,000 mg. dose given in the 16th and 17th weeks.

thenic acid to the four men who were deficient was followed by a prompt correction of the faulty eosinopenic response to ACTH and subsidence of most of the clinical symptoms. The erythrocyte sedimentation rate fell significantly in the antagonist pair. There was a coincidental slight rise in the serum cholesterol levels. A most striking response was the positivity of the nitrogen balance. Coenzyme A, which was given to the antagonist pair during the last week, produced no additional effects over those of pantothenic acid.

Especially difficult to interpret are the studies of gastric secretion. In earlier studies, complete histamine-fast achlorhydria developed. In this study, there was a transient fall in gastric secretory activity, followed by spontaneous recovery. It is possible that potassium deficiency or the metabolic alkalosis, or both, were responsible for this disorder of gastric function in earlier experiments. Pantothenic acid deficiency alone probably was not responsible.

Experiments such as these, long as rigidly confining tests, but short in man's life span, impress upon us the wide range of variation even in subjects chosen to conform to a standard. What Williams (41) has emphasized as biochemical individuality and Medawar (42) as the uniqueness of the individual, we see in the personal quirks and inconsistent biochemical patterns which bedevil the framers of human nutritional experiments and make exasperating the necessarily unsatisfactory solution of the problem of controls. We have no doubt that some of the discordant results among our experiments, or within a single test run, come from ignorance and poor planning, but the wide variations in normal persons can easily frustrate

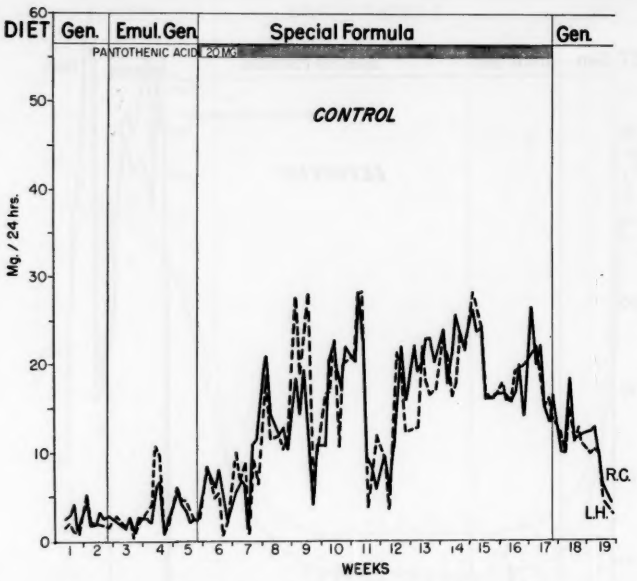

FIG. 20. URINARY EXCRETION OF PANTOTHENIC ACID (CONTROL)

When 20 mg. of pantothenic acid was given daily, the excretion rate averaged about the same.

the best experimental design if the requirement for pantothenic acid should vary manyfold in a small group of subjects.

Perhaps analagous to this individuality, accepted by the clinician but so troublesome to the investigator, is the subtle self-correcting tendency by which homeokinetic adjustments tend to bring the metabolic machinery back on the track after we force it off. We know little of metabolic detours and by-passes which can take over a threatened vital function, though some are recognized. Changes which occur during a period of unchanging experimental procedure seem well enough explained when abnormal signs, symptoms and metabolic function appear to be induced by a dietary deficiency or a deprivation of a specific vitamin. When these aberrations begin to correct themselves by something analagous to the "hunting reaction" of Lewis (43–45) our interpretation must be especially cautious for even the best controls leave much uncontrolled. This self-correcting tendency was a curious feature of some of the signs, symptoms and metabolic functions in our experiments. This emphasizes the need for cautious and critical interpretation of results. Symptoms of distress appeared, only to regress spontaneously with no change in the conditions of the experiment. When we increased the dose of antagonist from 750 to 1,000 mg. per day abnormalities reappeared. Perhaps functions so vital to much of the body's metabolic activity as those mediated by coenzyme A are protected by alternate metabolic pathways when pantothenic acid deficiency is becoming established. We have no data to explain the phenomena, nor clear ideas of how they are brought about.

Review of our errors in interpretation

Some of the abnormalities observed in earlier studies were caused by artifact and inadequacy in experimental design. We mistook these for signs and symptoms of pantothenic acid deficiency. The hypocholesterolemia undoubtedly resulted from the use of corn oil as the source of lipids in the diet (2, 4, 5). The abnormal glucose tolerance curves may have resulted from the rapid feeding of a liquid, partially hydrolyzed diet which could

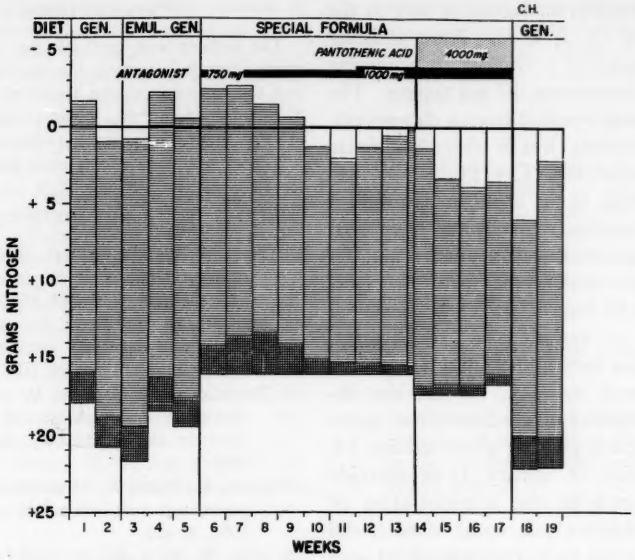

FIG. 21. NITROGEN BALANCE (ANTAGONIST GROUP)

A negative balance is shown by the shaded bar extending above the zero line, a positive balance below it. Administration of pantothenic acid starting the 14th week was followed by a markedly positive balance. This increased when a general diet was fed in the 18th week. The cross hatching indicates fecal nitrogen, the remainder of each bar represents urinary nitrogen, and the base of each bar represents dietary nitrogen.

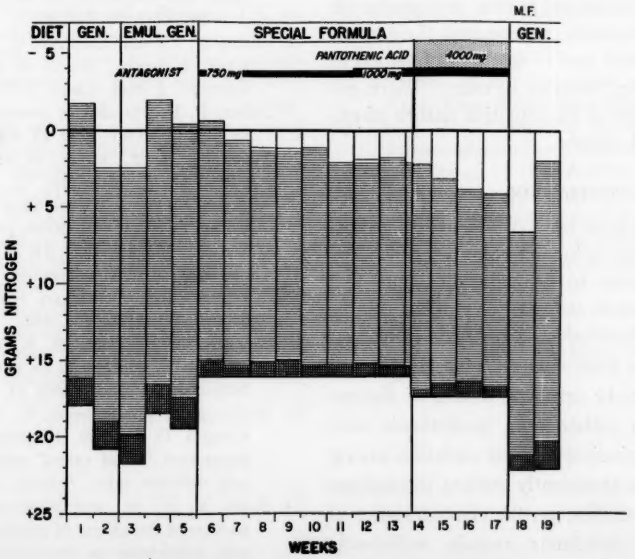

FIG. 22. NITROGEN BALANCE (ANTAGONIST GROUP)

be absorbed too rapidly, analagous in part to the dumping syndrome (1, 2, 4, 5). After omitting the hydrolysis employed previously in preparing the formula this phenomenon did not appear. The hypokalemic alkalosis resulted from a diet providing minimal potassium, but a disproportionate amount of bicarbonate ion (2, 4, 5).

We are not certain of the cause or nature of a few of the abnormalities. Some may have been caused by unrecognized flaws in experimental design. They might have come from the individual differences in response to a deficiency of pantothenic acid or variations in requirement. These abnormalities include the faulty acetylation of para-aminobenzoic acid (1, 2) and the development of arrhythmias, cardiovascular instability and electrocardiographic abnormalities (4, 5). The depression of urinary 17-ketosteroids probably was caused by the administration of large doses of PABA (1, 2, 4, 5) since it did not reappear when the dose was reduced. Large doses of PABA may have an inhibitory effect upon urinary excretion of 17-ketosteroids. The erratic abnormalities of water absorption and excretion in several subjects are not accounted for unless by varying motility.

Even with all precautions we may err in planning studies as well as in interpreting the results. Despite the slow and tedious course of such studies, their undramatic nature and their great cost we have no other way to assess the function of the less well known vitamins in man. There are few better examples of the tentative nature of experimental clinical science.

CONCLUSION

Further studies have been conducted on pantothenic acid deficiency in human volunteers, employing multiple controls to evaluate the signs and symptoms which arose in previous studies. Some of our previous observations have been fully confirmed, some have been reinterpreted, and a few have been found to be artifacts, results of the experimental design rather than pantothenic acid deficiency. The principle of great variation among normal people was abundantly evident throughout this and previous studies, yet the characteristics of pantothenic acid deficiency remain sufficiently clear and consistent to constitute an entity.

ACKNOWLEDGMENTS

The authors wish to thank the volunteers from Anamosa State Reformatory, the Warden, Mr. Ray Purcell, and the Chairman of the Board of Control, Mr. Robert C. Lappen, for their splendid cooperation. We also wish to express our thanks to the nurses and attendants whose conscientious efforts made possible this study.

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TOTAL NONDIALYZABLE SOLIDS (TNDS) IN HUMAN URINE. II. A METHOD FOR REPRODUCIBLE FRACTIONATION [1]

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The amount and nature of the total nondialyzable solids (TNDS) of normal human urine have been parametrically defined (1). This report is concerned with a technique for separation of the TNDS into three reproducible primary fractions, which are suitable for subfractionation by other methods, and their composition.

MATERIALS AND METHODS

Materials

Subjects. Twelve young adult individuals (seven male, five female), who had no history or clinical evidence of renal disease or current evidence of other disease, served as subjects for this study. Each subject continued the usual daily activities and diet during the collection of three 24 hour urine specimens. Female subjects submitted specimens near the midportion of the menstrual cycle.

Membranes for ultrafiltration. The solvent for collodion membranes was prepared from analytical grade reagents by mixing 96.1 ml. absolute ethanol, 40.5 ml. diethyl ether, and 13.5 ml. glacial acetic acid. One hundred ml. of collodion [2] was then added and thoroughly mixed.

Discs of the best grade of plate glass, 15 cm. in diameter, scratch-free, and hand-rubbed to provide a polished surface, were stored in bichromate cleaning solution. The edges of the glass plates were unbeveled. Prior to use, the plates were rinsed thoroughly with distilled water and air dried. The lint-free plate was then floated on mercury in a glass dish in a desiccator containing 75 ml. of concentrated sulfuric acid in the bottom. Exactly 12 ml. of the collodion solution was poured onto the center of the plate and allowed to dry for exactly 20 minutes in a closed desiccator. The collodion-covered disc was lifted out and slid into a vessel of distilled water. The gelled membrane floated free to the surface of the water and was transferred on a piece of filter paper for storage in distilled water saturated with thymol. The sulfuric acid was changed after preparation of each membrane.

These membranes have a "T" value of 14 to 34 seconds, *i.e.*, the period required for 100 ml. of distilled water to flow through 100 cm.^{2} of filtering area, at room temperature, at a differential pressure of 15 lbs. per sq. inch. They have a thickness of 100 μ, a dry weight of 1.4 mg. per cm.^{2}, and an average pore diameter of 20 (range. 14 to 26) mμ.[3] They are comparable in porosity to Schleicher and Schuell ultrafine filters, dense (average pore diameter, 10 to 20 mμ), but S. and S. ultrafine filters, medium (average pore diameter, 20 to 35 mμ) gave results comparable to those obtained with the membranes prepared in this laboratory.

Ultrafilters. Ultrafilters of 4 L. capacity, having porous steel supporting plates 15 cm. in diameter, were used.[4]

Methods

Fractionation and recovery. The collection and dialysis of 24 hour urine specimens was by the previously described technique (1). A 1/10 aliquot was removed for TNDS determination and the remainder of the 24 hour specimen was ultrafiltered at approximately 3° C., under a maximum pressure of 30 to 40 lbs. per sq. inch. This required about 24 hours. The solids in the ultrafiltrate were recovered by stepwise lyophilization, as previously described (1). The resultant brown powder was designated *UFO* (ultrafiltrate O). The residue, designated *RO* (residue O), was removed from the membrane and extracted three times with five volumes of barbital-sodium barbital buffer, ionic strength 0.1N, pH 8.6. The veronal insoluble fraction of *RO*, recovered by centrifugation (relative centrifugal force = 1,000 × G, 20 minutes) and designated as *R1* (residue 1), was immediately suspended in distilled water and dialyzed against cold distilled water for not less than 24 hours; lyophilization yielded a white, fluffy powder. The clear, brown supernatant barbiturate buffer extracts were pooled and dialyzed against cold distilled water to remove the buffer. A fluffy, tan solid, designated *RS1* (residue, soluble 1) was then recovered by lyophilization of the solution.

Analytical techniques. The lyophilized fractions were analyzed for ash, calcium, phosphorus, nitrogen, "sialic acid," hexose [5] and "bound" water as previously de-

[1] Supported by Mary Reynolds Babcock Foundation and Public Health Service Grants A-259 and H-2820, National Institutes of Health.

[2] Analytical reagent, Mallinckrodt Chemical Works.

[3] Characteristics of these membranes determined by Membranefiltergesellschaft, Goettingen, Germany, through the courtesy of Carl Schleicher and Schuell Company, Keene, N. H.

[4] Filter model 4000, F. R. Hormann & Co., Newark, N. J.

[5] The differences found in the quantity of hexose present in the respective fractions, as measured by the anthrone and orcinol reactions, are the consequence of us-

scribed (1). Qualitative spot tests (2) for various metals were made on samples of inorganic ash from each fraction. Hexosamine was determined by the following procedure. A 5 to 10 mg. sample in 2 ml. of water was placed in a 5 ml. glass ampul and 0.2 ml. of concentrated hydrochloric acid was added. The ampul was sealed and placed in boiling water for four hours. The hydrochloric acid was removed by evaporation in a current of air and the residue dissolved in water, adjusted to pH 9.8 with three to five drops of 1 per cent sodium hydroxide, and diluted with water to 10 ml. Aliquots were used for analysis as described by Schloss (3). The results are expressed in terms of glucosamine hydrochloride.

Hexuronic acid, expressed as glucuronic acid, was assayed by the method of DeFrates and Boyd (4). The samples were hydrolyzed in sealed ampuls with 6 N hydrochloric acid at 50° C. for three hours. The absorption curve of the reaction product paralleled that of the glucuronic acid standard. The optical density of the final color was maximal after a two hour hydrolysis and was the same after two and a half, three, and three and a half hour periods of hydrolysis. The sensitivity of the method could be increased to measure concentrations of hexuronic acid of less than 1 per cent by increasing the time of incubation with naphthoresorcinol. It is important that, when some samples were assayed by the modified carbazole method (5, 6), values which were obtained were similar to those with the above technique. In neither of these procedures was there a measurable hydrolytic loss of glucuronic acid. However, neither the commercial chondroitin sulfate, an authentic semipurified sample of the urinary aminopolysaccharide of Di Ferrante and Rich (14),[6] or a sample of the latter prepared by their technique (7) from our *UFO* fraction [12 per cent hexuronic acid (5)] gave a reaction with naphthoresorcinol under these conditions. Indeed, less than one per cent hexuronic acid was detected in a solution of 200 μg. per ml. of commercial chondroitin sulfuric acid,[7] and only 32 per cent of the theoretically present amount was detectable after hydrolysis for 12 hours at 80° C. Thus the method is not applicable to determinations of hexuronic acids present in acid mucopolysaccharides and the conjugate or conjugates measured by this method must be of another sort.

Total lipids, total phospholipids, total fatty acids and total unsaponifiable matter were determined as before (1) on material obtained by pooling equal quantities of each fraction from male and female subjects to form two pools, which were designated samples A and B. A sufficient quantity of material was thus obtained for complete lipid analysis. Total and esterified cholesterol were determined colorimetrically (8) on aliquots of the chloroform extracts, before and after precipitation of free cholesterol with digitonin. The values were corrected, assuming a light absorption for cholesterol esters 15 per cent greater than that for free cholesterol. This latter fraction was calculated as the difference between total and esterified cholesterol.

RESULTS

The TNDS from nine subjects, as determined from $\frac{1}{10}$ aliquots, gave a mean value of 505 (standard deviation ± 105) mg. per 24 hours, an increase of 17 per cent above our previously reported value (1). The present collection period covered the months of September to November, whereas previous studies were from January to March.

Recovery of TNDS following fractionation

Eighty-two (range, 70 to 87) per cent of the TNDS of each subject was accounted for as the sum of *R1, RS1* and *UFO*. One-fourth of the loss was accounted for by the entrapment of 4 to 5 per cent of the TNDS in the pores of the ultrafilter membrane. This is presumably a mixture of *R1* and *RS1,* and can be determined by solution of the membranes in half and half ethanol-ether solution. The "bound" water content of the sum of the individual fractions averaged 21 mg. per 24 hours less than that of the TNDS. Loss of any portion of this water during the lyophilization of the fractions would appear as unrecovered TNDS in the calculations of recovery. The remainder of the loss during fractionation is prob-

ing as a standard a mixture of galactose and mannose (1:1, by wt.). Such a standard does not adequately match the more complex, but unknown, pattern of polysaccharide content of these materials. Galactose, mannose, fucose, rhamnose and glucose are found chromatographically in each of these three fractions. The optical densities of 10 mg. per 100 ml. solutions of these sugars by the orcinol methods, measured with 1 cm. cuvettes in the Beckman DU spectrophotometer, were 0.138, 0.122, 0.100, 0.098 and 0.026, respectively. For 5 mg. per 100 ml. solutions, by the anthrone methods, they were 0.435, 0.366, 0.615, 0.600 and 0.710, respectively. Obviously, the two series are roughly reversed with respect to color intensity. Since chromatography suggests that galactose and mannose are the predominant sugars in each fraction, the true values for hexose probably lie between those given for the two methods, and are nearer to those obtained by the orcinol method. The anthrone/orcinol ratio is thus an important indication of qualitative variation in hexose content, and is useful for comparison of the three fractions (Table VIII).

[6] Supplied by Dr. Clayton Rich, The Rockefeller Institute for Medical Research. Sample assayed 15 per cent hexuronic acid (5), 17.4 per cent hexosamine.

[7] Contained 25.1 per cent hexosamine and 24.5 per cent hexuronic acid (5). General Biochemicals, Inc. This material was ultrafiltrable.

ably mechanical, since none of the fractions were dialyzed after lyophilization (*vide infra*).

Possible alterations of TNDS by the fractionation procedure

Several experiments were designed to investigate the possibility that the separation procedure might produce alterations in TNDS such that the fractions would not accurately reflect the native state of these materials.

When clear whole urine was ultrafiltered, then dialyzed and again ultrafiltered, nothing was found on the filter membrane after the second ultrafiltration. This indicated that dialysis *per se* did not cause a coalescence of molecules to produce *RO*. When the urine was ultrafiltered directly, without preliminary dialysis, approximately the same *RS1/RO* ratio (0.29) was found as with dialyzed urine (0.3 to 0.4 in four large pools), a finding which suggests that the proportion of these materials in undialyzed urine is the same.

Addition to *UFO* of sodium chloride to 0.58 N at 3° C. did not induce any coalescence into nonultrafiltrable material on standing.

In another experiment, a three L. sample of whole urine was divided into three equal parts. To one was added 30 ml. of a solution containing 141 mg. *RS1;* to the second was added 2 ml. of serum containing 169 mg. of nondialyzable material; the third was the control. These samples were fractionated as usual. After correction for the usual 18 per cent mechanical loss, the recoveries of the added *RS1* and serum were 76 and 89 per cent, respectively. Most of the missing *RS1* can be accounted for, since the added *RS1* was made up from lyophilized material (see below). These results suggest that there was no marked change in the solubility of either the *RS1* or the serum proteins, and therefore that *R1* is not a denaturation product of *RS1,* or *vice versa.* Other evidence for this is (*a*) the small but statistically significant chemical differences in the two fractions; (*b*) the constancy of the *RS1/RO* ratio at a given season of the year (in pooled samples from a variety of normal subjects this ratio agreed within 5 per cent); (*c*) the continued solubility of *RS1* after repeated freezings and lyophilization, in contrast to *R1,* which becomes highly water insoluble on lyophilization; and (*d*) in experiments to be described in detail elsewhere, various clinical conditions have been studied in which either *R1* or *RS1* was quantitatively changed, without significant alteration from normal in the other fraction.

Quantitative data

The results from analyses of the three fractions are presented in Tables I through V. The composition of each fraction is summarized in Table VI, and the 24 hour excretion rates are presented

TABLE I

Composition of veronal insoluble fraction (R1) of normal urinary TNDS, expressed as per cent of dry weight*

Subject no.	Dry weight	Hexosamine		Hexose			Sialic acid	Ash	"Bound" water	Nitrogen	Ca†	P†
		Female	Male	Anthrone	Orcinol	Anth./Orc.						
	mg./24 hrs.											
1	51	5.0		14.3	8.1	1.83	6.3	5.7	5.3	8.8	3.4	6.7
2	168	4.4		11.3	7.2	1.57	2.6	5.8	5.4	9.2	11.5	22.5
3	72		6.2	12.0	7.7	1.56	2.6	4.5	8.7	10.0	6.8	24.3
4	131		6.1	17.3	12.3	1.41	2.5	5.2	6.8	10.4	8.4	18.9
5	88		6.0	11.9	9.6	1.24	3.6	5.9	6.7	11.2	5.3	9.3
6	78	3.0		15.4	6.7	2.30	0.0	4.7	7.1	11.2	7.1	33.5
7	137	4.3		14.8	6.3	2.35	0.0	4.5	5.3	11.4	7.0	31.4
8	108	4.0		11.2	5.4	2.06	3.5	3.8	5.2	11.4	6.9	26.0
9	35		7.2	13.0	6.9	1.88	8.3	3.2	6.0	9.7	3.1	
10	69		5.2		8.3		0.0	6.1	5.8	10.6	26.0	10.2
11	88		6.6		9.7		3.1	3.8	6.7	10.2	14.5	30.1
12	52		6.4		8.1		9.7			10.1		
Mean	89.6	4.14	6.24	13.52	8.03	1.800	3.52	4.84	6.27	10.35	9.09	21.30
S.E. mean	11.42	0.33	0.23	0.76	0.53	0.129	0.91	0.30	0.23	0.25	2.06	3.06

* Total nondialyzable solids.
† Per cent of ash.

TABLE II

Composition of veronal soluble fraction (RSI) of normal urinary TNDS, expressed as per cent of dry weight

Subject no.	Dry weight	Hexosamine		Hexose			Sialic acid	Ash	"Bound" water	Nitrogen	Ca*	P*
		Female	Male	Anthrone	Orcinol	Anth./Orc.						
	mg./24 hrs.											
1	29	7.4		11.9	9.9	1.20	6.6	3.3	6.6	9.1	12.6	7.5
2	65	4.5		14.2	9.6	1.48	1.9	2.4	10.3	9.6	12.8	48.8
3	22		5.7		8.1		2.4	5.4	12.5	9.7	5.3	14.4
4	72		4.6	10.8	7.5	1.44	1.9	4.3	5.8	8.9	8.3	31.6
5	44		6.9	11.8	8.6	1.37	5.2	3.4	10.3	9.7	5.7	22.7
6	124	5.1		10.9	7.1	1.54	2.9	5.0	9.4	9.9	6.2	8.0
7	60	3.6		9.0	6.4	1.41	1.6	8.2	8.2	10.9	2.8	7.3
8	65	10.2		18.1	14.2	1.27	10.5	8.0	12.5	9.4	3.8	12.1
9	58		5.6	9.0	6.7	1.34	9.0	8.3	9.6	10.7	2.4	1.2
10	64		7.0		10.6		5.5	7.2	1.8	9.9	16.0	10.8
11	49		6.3		7.7		12.4	9.2†	6.9†	9.4	4.1†	6.7†
12	54		6.1		7.1		13.2			9.4		
Mean	58.8	6.16	6.03‡	11.96	8.63	1.381	6.09	5.88	8.54	9.72	7.27	15.55
S.E. mean	7.34	2.23	0.31	1.06	0.64	0.049	1.23	0.72	0.95	0.17	1.38	4.18

* Per cent of ash.
† Approximately equal weights of protein of numbers 11 and 12 combined for these determinations.
‡ The combined mean for female and male = 6.08 ± 0.50 per cent.

in Table VII. The significant differences between the three fractions are summarized in Table VIII.

Two-dimensional chromatograms of the undialyzed materials, using the three previously reported solvent systems, revealed the presence in each of the three fractions of all those amino acids previously reported to be present in the TNDS (1). By this technique, it was not possible unequivocally to distinguish any one of the three fractions from the others. The amino acids demonstrated in these studies of course occur ubiquitously.

The previous observation (9) that males normally excrete more nondialyzable hexosamine per 24 hours than females has been confirmed in the present studies, and the increase is primarily in the *UFO*.

Hexuronic acid was also found predominantly

TABLE III

Composition of ultrafiltrate (UFO) fraction of normal urinary TNDS, expressed as per cent of dry weight

Subject no.	Dry weight	Hexuronic acid	Hexosamine		Hexose			Sialic acid	Ash	"Bound" water	Nitrogen	Ca*	P*
			Female	Male	Anthrone	Orcinol	Anth./Orc.						
	mg./24 hrs.												
1	221	1.2	8.1		27.4	15.9	1.72	11.0	20.8	7.5	6.1	6.1	5.4
2	189	1.1	9.3		39.3	16.4	2.40	7.1	21.4	6.7	5.2	12.1	3.6
3	208	1.4		11.6	21.0	16.6	1.27	8.5	22.2	5.7	4.8	12.8	4.5
4	217	1.7		12.4	20.6	18.5	1.11	8.8	18.9	7.3	5.3	11.8	3.7
5	246	0.8		12.1	18.5	15.1	1.23	13.2	14.8	8.4	5.8	8.0	9.0
6	200	1.4	9.5		17.4	13.6	1.28	8.3	24.0	7.4	4.6	12.0	5.2
7	146	0.8	9.8		18.7	14.6	1.28	12.5	22.2	8.6	6.1	10.6	6.2
8	259	2.8	6.6		12.4	13.2	0.94	9.7	30.9	6.6	4.5	8.3	10.2
9	522	2.2		8.3	19.0	13.5	1.41	12.3	20.2	9.1	9.2	5.0	11.2
10	324	0.9		7.0		11.8		8.7	16.4	7.9		11.5	8.5
11	360	0.8		10.6		18.6		14.9	16.9	10.1		10.4	5.7
12	292	0.8		10.9		14.6		11.6	18.2	8.2	5.1	9.0	7.6
Mean	264.5	1.33	8.66	10.41†	21.59	15.20	1.40	10.55	20.58	7.79	5.67	10.08	6.73
S.E. mean	29.17	0.18	0.59	0.77	2.57	0.60	0.14	0.69	1.22	0.35	0.43	0.65	0.73

* Per cent of ash.
† Difference between female and male has p of $<0.2>0.1$. Combined mean = 9.68 ± 0.55 per cent.

TABLE IV

Lipid composition of the ultrafiltrate (UFO), veronal soluble (RSI), and veronal insoluble (RI) fractions of normal urinary TNDS, expressed as per cent of dry weight of each fraction

Sample*	Fraction	Total lipids†	Total phospho-lipids‡	Total fatty acids	Unsaponifiable			
					Total	Cholesterol		
						Total	Free	Ester
	RI	5.60	1.64	3.54	2.24	0.61	0.48	0.13
	RSI	15.10	3.27	3.74	4.79	1.02	0.74	0.28
A	weighted average§	8.45	2.13	3.60	3.00	0.73	0.55	0.18
	UFO	1.01	0.08		0.10			
	RI	11.15	2.82	4.91	1.50	1.09	0.74	0.35
	RSI	10.00	1.29	1.50	6.13	0.76	0.47	0.29
B	weighted average§	0.80	2.36	3.89	2.89	0.99	0.66	0.33
	UFO	0.43	0.05		0.03			

* Combined samples from several subjects.
† Weight of the chloroform extract.
‡ Lipid P × 25.
§ Figures recalculated assuming average values of 0.7 and 0.3 for the ratios *RI/RO* and *RSI/RO*, respectively.

in the *UFO* fraction. The *RSI* fraction has one-half per cent or less. None was detected in the *RI* fraction.

Paper electrophoresis with toluidine blue-O staining of the three fractions at pH 8.6 revealed the consistent presence of material staining metachromatically in the *UFO* fraction. Although there was considerable "trailing," the migration of this material was similar to that of chondroitin sulfate. A very small quantity of material staining metachromatically and of similar mobility was usually evident in the *RSI* fraction. There was no metachromasia in any of the *RI* preparations.

DISCUSSION

The present report is concerned with the parametric composition and reproducibility of the fractions rather than with the identification of individual components of TNDS. The residue *RI* is composed of the Tamm and Horsfall mucoid ("uromucoid") (10, 11). The *RSI* fraction has been studied electrophoretically and includes serum proteins (12). The *UFO* consists of heterogeneous groups of molecules which include the "acid mucopolysaccharide" of Kerby (13) and Di Ferrante and Rich (14).

TABLE V

Some cations detected in ash of veronal insoluble (RI), veronal soluble (RSI) and ultrafiltrate (UFO) fractions of normal urinary TNDS

	Al	Ni	Zn	Pb	Fe^{+++}	Fe^{++}	Mg
RI	5/7*	9/9	8/9	6/6	7/9	5/9	3/3
RSI	0/6	10/10	9/10	7/8	3/11	5/11	3/3
UFO	10/16	16/16	16/16	15/16	4/16	11/16	11/11

* Number samples giving positive test per number of samples tested.

Inorganic constituents

The lack of a consistent calcium/phosphorus ratio in the various fractions is to be expected, since tests for inorganic phosphorus were nega-

TABLE VI

Average composition of veronal insoluble (RI), veronal soluble (RSI), and ultrafiltrate (UFO) fractions of normal urinary TNDS, expressed in per cent of dry weight

	RI	*RSI*	*UFO*
Hexuronic acid		0.50	1.33
Hexosamine	5.19	6.08	9.68
Hexose*	10.78	10.30	18.40
Sialic acid	3.52	6.09	10.55
Protein†	61.69	56.70	29.00
Lipid	8.37	12.55	0.72
Ash	4.84	5.88	20.58
"Bound" water	6.27	8.54	7.79
Total	100.66	106.64	98.05

* Average of values obtained with anthrone and orcinol methods.
† N × 6.25, corrected for N in sialic acid and hexosamine.

TABLE VII

Rate of excretion for the constituents of three fractions of normal urinary TNDS, expressed as mg. per 24 hours

Constituent	Mean *RI**	± S. E. *RI*	Mean *RSI**	± S. E. *RSI*	Mean *UFO**	± S. E. *UFO*
Total	89.6	11.42	58.8	7.34	264.5	29.17
Hexuronic acid					3.55	0.84
Hexosamine (female)	4.50	0.97	4.02	1.00	17.20	0.78
Hexosamine (male)	4.71†	0.70	3.11†	0.36	31.0†	2.83
Hexosamine (female and male)	4.63	0.55	3.49	0.46		
Hexose (anthrone)	13.03	2.08	7.70	1.25	51.36	7.63
Hexose (orcinol)	7.23	1.11	5.02	0.67	39.94	4.23
Sialic acid	3.38	0.31	3.38	0.69	28.93	4.49
Ash	4.58	2.32	3.54	0.57	53.60	5.95
"Bound" water	5.75	0.67	5.02	0.90	21.33	3.12
Nitrogen	9.44	1.15	5.82	0.71	15.22	3.71
Calcium	0.44	0.11	0.23	0.06	5.10	0.33
Phosphorus	3.95	0.89	0.43	0.09	3.95	0.89

* *RI:* veronal insoluble fraction. *RSI:* veronal soluble fraction. *UFO:* Ultrafiltrate.
† p for difference between female and male for *RI:* $<0.9>0.8$, for *RSI:* $<0.5>0.3$, for *UFO:* $<0.01>0.001$.

tive prior to ashing the samples. The presence of those cations indicated in Table V were specifically demonstrated by the spot tests and confirmed in most instances by spectrography. Tests for copper and manganese were negative. The heavy-metal ions found are probably adventitious, attributable to the metal-binding capacity of proteinaceous materials present in the fractions. The absence of copper suggests that ceruloplasmin is not a prominent constituent of the TNDS.

TABLE VIII

Comparison of per cent composition of three fractions of normal urinary TNDS

Constituent	Number *RI**	Mean *RI*	Number *RSI**	Mean *RSI*	Number *UFO**	Mean *UFO*	Statistical comparisons: t_1 *RI* with *RSI*	p t_1	t_2 *UFO* with *RSI*	p t_2	t_3 *UFO* with *RI*	p t_3
Hexosamine (female)	(5)	4.14	(5)	6.16	(5)	8.66	†					
Hexosamine (male)	(7)	6.24	(7)	6.03	(7)	10.41	†					
Hexosamine (female and male)			(12)	6.08	(12)	9.68			4.836	0.001		
Hexose (anthrone)	(9)	13.52	(8)	11.96	(9)	21.59	1.248	$<0.3>0.2$	3.310	$<0.01>0.001$	3.028	$<0.01>0.001$
Hexose (orcinol)	(12)	8.03	(12)	8.63	(12)	15.20	0.724	0.5 0.3	7.491	0.001	8.905	0.001
Hexose (anth./orc.)	(9)	1.800	(8)	1.381	(9)	1.40	2.940	0.02 0.01	0.127	0.9	2.076	0.1 0.05
Sialic acid	(12)	3.52	(12)	6.09	(12)	10.55	1.682	0.2 0.1	3.168	0.01 0.001	5.004	0.001
Ash	(11)	4.84	(11)	5.88	(12)	20.58	1.337	0.2 0.1	10.131	0.001	12.024	0.001
"Bound" water	(11)	6.27	(11)	8.54	(12)	7.79	2.333	0.05 0.02	0.770	0.5 0.3	3.665	0.01 0.001
Nitrogen	(12)	10.35	(12)	9.72	(10)	5.67	2.100	0.05	9.330	0.001	9.811	0.001
Calcium	(11)	9.09	(11)	7.27	(12)	10.08	0.759	0.5 0.3	1.896	0.1 0.05	0.497	0.7 0.6
Phosphorus	(11)	21.30	(11)	15.55	(12)	6.73	1.090	0.3	2.170	0.05 0.02	5.038	0.001

* *RI:* veronal insoluble fraction. *RSI:* veronal soluble fraction. *UFO:* Ultrafiltrate.
† For *RI* the probability (p) for the difference between female and male = <0.001. Therefore no comparison was run with *RSI*.

Protides

When the nitrogen content of each fraction is corrected for the nitrogen content of hexosamine and sialic acid and then multiplied by the factor 6.25, the *R1, RS1* and *UFO* are shown to contain 55, 33 and 77 mg. per day of protein material, respectively. Correction of this sum for the overall loss of 18 per cent incurred during fractionation gives a protein excretion of 201 mg. per 24 hours, which is in agreement with the previously determined (1) value of 204 mg. per 24 hours for the TNDS.

It is of interest that the TNDS account for less than one-third of the total "bound" amino acids of human urine (15–17). It would appear that two-thirds of the "bound" amino acids of normal urine are of such dimensions as to be dialyzable through cellophane membranes.

Glucides

Since a chondroitin sulfate has been found in urine (13, 14), the results obtained by the two methods, taken together, indicate the presence of one or more nondialyzable hexuronic acid conjugates other than chondroitin sulfate.

Lipids

The lipids represent only a small portion of the dry weight of the ultrafiltrate (1 per cent or less). On the other hand, the total nonultrafiltrable residue contains sizeable amounts of lipids (as much as 10 per cent). A recalculation of the values of this material indicates a lipid composition very similar in the two pooled samples which were analyzed. In both samples, all types of lipids are distributed between the *R1* and the *RS1* fractions. However, the distribution of the individual lipids in sample A is quantitatively rather different from that found in sample B.[8]

[8] The separation of the *R1* and *RS1* fractions prior to the lipid analysis has been carried out on pooled samples of *RO* which had been preserved in the frozen state. It cannot be excluded that some splitting of the linkages between the protein and lipid moieties of lipoproteins might have occurred during the freezing and thawing of these samples, and that this might account, in part at least, for the differences observed (18) between samples A and B.

Comparison of the composition of the three fractions

Referring to Table VIII, one will note certain differences in the percentage composition of the three fractions which are significant if one accepts a probability of < 0.05. The hexosamine constant of *R1* was significantly less in females, whereas there was no sex difference in either *RS1* or *UFO*. The ratio for hexose (orcinol/anthrone) in *R1* was significantly greater than in *RS1*. The "bound" water content of *RSI* was greater than for *R1*, while the nitrogen content of *R1* exceeded that for *RS1*. Also, it will be seen that *UFO* differs significantly from *RS1* and *R1* except with respect to the hexose ratio, "bound" water, and calcium for the former and hexose ratio and calcium for the latter.

Two additional observations made during this work may be useful to other workers.

First, concentration of *UFO* by dialysis through Visking® cellulose tubing against concentrated polyvinylpyrrolidone[9] or polyethylene glycol[10] was not satisfactory, since a small but significant proportion of the polymer passed through the membrane and became mixed with the sample.

Second, in experiments with two specimens, lyophilization of TNDS was found to cause 69 and 48 per cent of it to become dialyzable. The material which passed through the cellophane was strongly ninhydrin positive, did not contain sialic acid or hexose, and was a hygroscopic gum on relyophilization. The major contributor to this loss is the *UFO* fraction, since, when lyophilized samples of the three fractions were similarly checked, the percentages of the starting weights lost by dialysis were respectively 83, 3 and 6 for *UFO, RS1* and *R1*. Moreover, the naphthoresorcinol-determined hexuronic acid (but not that determined with carbazole, which was correspondingly concentrated), part of the hexose and of the sialic acid was dialyzable from the *UFO*, showing that the fractions may not behave in a manner comparable to the TNDS under these conditions. These observations demonstrate that lyophilization may have drastic effects on these materials and that redialysis may result in loss.

[9] Plasdone C®, Antara Chemicals, General Aniline and Film Corp.

[10] Carbowax 6000®, Union Carbide Chemicals Co.

SUMMARY

A method has been described for the separation of urinary total nondialyzable solids (TNDS) into three reproducible fractions. The 24 hour excretion rates and the overall chemical composition of these three fractions is presented.

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